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**Modulation of Cholinergic Synaptic Transmission  
in an Identified Locust Sensory Pathway**

**Sarah Jane Judge**

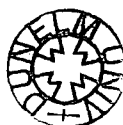
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**Department of Biological Sciences**

**University of Durham**

**2000**



20 MAR 2001

**Sarah Jane Judge**

**Modulation of Cholinergic Synaptic Transmission in a Locust Sensory Pathway.**

**Submitted for the Degree of Ph.D. 2000.**

**Abstract**

The monosynaptic connection between the locust forewing stretch receptor (fSR) and the first basalar motoneuron (BA1) is part of a sensory pathway involved in flight. The fSR is a single sensory afferent, triggered during wing elevation, that makes central connections with the wing depressor motoneuron, BA1. The fSR/BA1 synapse in *Locusta migratoria* was used as a model to study the modulation of cholinergic synaptic transmission.

Electrophysiological experiments indicate that presynaptic muscarinic cholinergic receptors are involved in the down-regulation of acetylcholine release from the fSR terminals and at least some of these are located on and activate GABAergic interneurons that inhibit the fSR. These experiments are supported by electron microscopical (EM) immunocytochemical (ICC) studies, which show that the fSR receives synaptic inputs from neurons immunoreactive (IR) for GABA.

Additional EM ICC studies reveal that the fSR also receives synaptic inputs from glutamate-IR neurons. The EM ICC studies also showed that neurons that are not immunoreactive for GABA or glutamate are presynaptic to the fSR and its postsynaptic member, suggesting that the fSR/BA1 synapse is modulated by other neuromodulators. Further electrophysiological studies revealed that the biogenic amines, octopamine and dopamine are potentially capable of modulating the fSR/BA1 synapse, by suppressing the postsynaptic response of BA1 to ACh (at least in part). The biogenic amine, 5-hydroxytryptamine (5HT) is also potentially capable of modulating the fSR/BA1 synapse but may act through octopamine receptors. This is supported by confocal microscopical ICC studies, which show that the neuropil region containing the fSR/BA1 synapses is octopamine-IR but not 5HT-IR. These results indicate that a range of neuromodulators acting via different mechanisms interact to modulate cholinergic synaptic transmission between the fSR and BA1.



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## **Declaration**

The work presented in this thesis was carried out by me and is an accurate record of the experimental work undertaken by me. This work, under the supervision of Dr. Beulah Leitch, has been carried out in the Department of Biological Sciences, at the University of Durham, and has not been previously submitted for a higher degree.

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## **In Press**

The majority of the results presented in Chapter 3 have been published in, Judge, S. and Leitch, B. (1999c) Modulation of transmitter release from the locust forewing stretch receptor neuron by GABAergic interneurons activated via muscarinic receptors. *J. Neurobiol.*, 40:420-431.

The majority of the results presented in Chapter 4 have been published in, Judge S. and Leitch, B. (1999) GABA-immunoreactivity in processes presynaptic to the locust wing stretch receptor neuron. *J. Comp. Neurol.*, 407:103-114.

For my mother and father, with love.

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## Glossary

ACh	acetylcholine
Ag	silver
AK	adipokinetic
aLAC	anterior lateral association centre
ATP	adenosine triphosphate
aVAC	anterior ventral association centre
BA1	first basalar motoneuron
Ca <sup>2+</sup>	calcium
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine monophosphate
ChAT	choline acetyltransferase
Cl <sup>-</sup>	chloride
CNS	central nervous system
CPG	central pattern generator
DAB	diaminobenzidine
DDW	deionised distilled water
D <sub>f</sub>	fast coxal depressor
DIT	dorsal intermediate tract
DMT	dorsal median tract
DUM	dorsal unpaired median
EM	electron microscopical
EPSP	excitatory postsynaptic potential
FeCO	femoral chordotonal organ



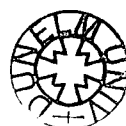
FETi	fast extensor tibiae
fSR	forewing stretch receptor
GABA	$\gamma$ -aminobutyric acid
GPR	gastropyloric receptor
G-protein	GTP-protein
GTP	guanosine triphosphate
HRP	horseradish peroxidase
hSR	hindwing stretch receptor
Hz	hertz
I	current
ICC	immunocytochemistry
IPSP	inhibitory postsynaptic potential
IR	immunoreactive
K <sup>+</sup>	potassium
LDT	lateral dorsal tract
LG	lateral giant neuron
LGMD	lobula giant movement detector
IVAC	lateral association centre
M	molar
MDT	median dorsal tract
ml	millilitre
mM	millimolar
mS	millisecond
mV	millivolt
$\mu$ l	microlitre

$\mu\text{m}$	micrometre
$\mu\text{s}$	microsecond
N	nerve
nA	nanoampere
$\text{Na}^+$	sodium
NCC	nervi corporis cardiaci
PAD	primary afferent depolarisation
PD	pyloric dilator
R	resistance
SETi	slow extensor tibiae
SR	stretch receptor
V	volt
VAC	ventral association centre
VD	ventral dilator
vGI	ventral giant interneuron
VIT	ventral intermediate tract
vVAC	ventralmost ventral association centre
5HT	5-hydroxytryptamine

## Chapter 1. General introduction

It was first proposed, at the end of the nineteenth century, that neurons communicate with each other via specialised junctions called synapses (Sherrington, 1897). It has since been widely accepted that transmission at these synapses is either chemical (Langley, 1906; Dale, 1935; Katz, 1969) or electrical (Furshpan and Potter, 1957). Chemical synapses are far more common than electrical synapses, which may be explained by the fact that transmission at chemical synapses can be modulated, whereas the effectiveness of transmission at electrical synapses is less susceptible to change.

Modulation of synaptic transmission may change the effectiveness of certain neuronal pathways which contributes significantly to the survival of the animal. For example, there is evidence that a cockroach ignores the stimulation of filiform hairs caused by its own movement but responds to wind stimulation of the hairs because synaptic transmission from the filiform afferents is inhibited during its own movement (Plummer and Camhi, 1981; Boyan, 1988). The modulation of synaptic transmission is also important for higher functions such as learning and memory as it allows the animal to acquire and store knowledge about the environment to aid it in the future (Kandel and Schwartz, 1982; Hasselmo, et al., 1995, 1996). For example, stimulating the tail of *Aplysia* enhances the defensive withdrawal reflex of the gill and siphon in response to siphon stimulation i.e. the animal learns to strengthen its defensive reflexes after receiving a potentially harmful stimulus to the tail (Castellucci and Kandel, 1976). This sensitisation is the result of the tail stimulus



facilitating synaptic transmission between the siphon sensory neurons and the motoneurons in the gill and siphon withdrawal reflex.

Therefore, an animal's survival is dependent on its adaptation to the changing environment and this adaptation can be achieved by modifying the strength of certain neuronal pathways through modulating chemical synaptic transmission

### **1.1. Synaptic transmission**

To see how transmission at a chemical synapse can be modified the processes underlying chemical synaptic transmission have to be understood. Chemical synaptic transmission can be separated into two main processes; the release of molecules of chemicals called neurotransmitters from a presynaptic neuron into an extracellular space called a synaptic cleft, and the activation of receptors on the postsynaptic neuron which evokes a response.

Neurotransmitters are released from a neuron when a depolarising action potential arrives at its presynaptic terminals. The action potential, which depends upon sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ ) channels, opens voltage-gated calcium ( $\text{Ca}^{2+}$ ) channels in the presynaptic terminals (Baker et al., 1971) which causes an influx  $\text{Ca}^{2+}$  ions (Katz and Miledi, 1967). The  $\text{Ca}^{2+}$  ions bind with transmitter release-associated proteins e.g. synaptotagmin (Littleton et al., 1994; Kelly, 1995; Littleton and Bellen, 1995), which stimulate the synaptic vesicles to discharge their neurotransmitter molecules by exocytosis. The calcium channels, transmitter release-associated proteins and synaptic vesicle release sites are thought to exist as a multimolecular entity (see Stanley,

1997), based on the evidence that the  $\text{Ca}^{2+}$  ions influx can trigger neurotransmitter release within 0.2 ms (Llinás et al., 1981). The synaptic vesicles each release a relatively fixed number of neurotransmitter molecules known as a quantum of neurotransmitter, which produces a unit potential in the postsynaptic neuron of a fixed size (Fatt and Katz, 1952). The more quanta of neurotransmitter released, the larger the potential in the postsynaptic neuron. Therefore synaptic transmission and the size of the potentials in the postsynaptic neuron can be enhanced or reduced by altering the number of quanta released.

Having crossed the synaptic cleft the neurotransmitter molecules are recognised and bound by specific receptors on the postsynaptic neuron. The receptors then mediate a response in the postsynaptic neuron by gating an ion channel. The response that the receptors mediate depends upon the type of ion channel they gate. For example, activation of GABA and glycine receptors, which gate chloride ( $\text{Cl}^-$ ) channels, results in an inhibitory response in the postsynaptic neuron (Coombs et al., 1955; Hamill et al., 1983; Jackel et al., 1994).

The receptors that have an integral ion channel are known as ionotropic receptors. They are made up of a number of transmembrane protein subunits that form an ion channel in the cell membrane with a neurotransmitter recognition site on the outer surface of the cell. Activation of the recognition site results in a conformational change in the protein subunits, which opens the ion channel. Receptor types which belong to this group include the nicotinic cholinergic receptor (Raferty et al., 1980; Changeux et al., 1984; Hanke and Breer, 1986), the  $\gamma$ -aminobutyric acid (GABA) receptor (Olsen and Tobin, 1990; Ortells and Lunt, 1995; Hosie et al., 1997), the

glycine receptor (Betz et al., 1993; Ortells and Lunt, 1995) and some glutamate receptors (Hollman et al., 1989; Keinänen et al., 1990; Cully et al., 1996). Recent advances in molecular techniques have allowed these ionotropic receptors to be sequenced and have shown that the receptors share a high degree of amino-acid sequence similarities, indicating they are phylogenetically related (Betz, 1990; Ortells and Lunt, 1995; Xue, 1998).

Receptors which gate ion channels not integral with the receptor itself are known as metabotropic receptors and consist of a single protein with seven membrane-spanning regions. The receptors possess a neurotransmitter recognition site just within the intracellular bilayer of the cell, and are coupled to a guanosine triphosphate (GTP)-binding protein (G-protein). Activation of the metabotropic receptor may stimulate the G-protein to trigger an effector enzyme (e.g. adenylate cyclase or phospholipase) to form intracellular second-messengers (e.g. cyclic adenosine 3',5'-phosphate [cAMP] or inositol phosphate) that either activate protein kinases to phosphorylate the ion channels (Klein et al., 1982; Siegelbaum et al., 1982; Abrams et al., 1984; Shuster et al., 1985) or modulate the ion channels themselves (Fesenko et al., 1985).

Types of receptor which belong to this group include the muscarinic cholinergic receptor (Kubo et al., 1986; Felder, 1995; Trimmer and Qazi, 1996), the dopamine receptor (Nathanson and Greengard, 1973; Bodnaryk, 1979; Deterre et al., 1982; Uzzan and Dudai, 1982; Stoof et al., 1984; Downer et al., 1985; de Vlieger et al., 1986; Pratt and Pryor, 1986; Orr et al., 1987), the octopamine receptor (Nathanson and Greengard, 1973; Uzzan and Dudai, 1982; Orchard et al., 1983; Orr and Hollingworth, 1990; Orr et al., 1991; Kaupp et al., 1992; Lange and Tsang, 1993; Baines and Downer, 1994; Li et al., 1994) the adrenergic receptor (Madison and Nicoll, 1986; Fraser et al., 1989) and

a glutamate receptor (Masu et al., 1991). Examination of the amino-acid sequences of the receptors in this superfamily not only demonstrates that the G-protein-coupled receptors share a high degree of sequence similarity with a characteristic motif of seven membrane-spanning regions but reveals how they evolved from a single receptor type to bind different ligands and activate different second messenger systems (Fryxell, 1995).

As the numerous events underlying chemical synaptic transmission, described above, occur every time one neuron communicates with another via a chemical synapse, one of the main disadvantages of chemical synaptic transmission is the delay caused by the numerous stages involved in releasing the neurotransmitters and evoking a postsynaptic response. However, one of the main advantages of chemical synaptic transmission is that the effectiveness of transmission can be modified at one or more of the stages.

## **1.2. Neuromodulators**

Like chemical synaptic transmission itself, the modulation of chemical synaptic transmission is undertaken by chemical molecules. However, the chemical molecules involved in modulating synaptic transmission are usually referred to as neuromodulators. Unlike neurotransmitters, neuromodulators do not necessarily excite or inhibit the neuron on which they act, but alter the cellular properties of the neuron to modify the transmission of the neurotransmitter.

Confusingly, many chemicals that are neuromodulators are also neurotransmitters. These include acetylcholine (ACh) and the amino-acids, GABA and glutamate as well as the biogenic amines, 5-hydroxytryptamine (5HT, also known as serotonin), 3-hydroxytyramine (also known as dopamine) and octopamine. The effects of each of the above mentioned neuromodulators on synaptic transmission and behaviour are discussed in detail in the following chapters but an overview of how neuromodulators as a whole modify synaptic transmission is given below.

### **1.3. Modulation of synaptic transmission**

The modulation of synaptic transmission not only depends on which neuromodulators are involved but also depends on the receptors on which they act and on which neuron the receptors are located. Receptors located on the presynaptic neuron are involved in altering the quanta of neurotransmitter released from the presynaptic neuron and receptors located on the postsynaptic neuron are involved in modifying the response of the postsynaptic neuron to the neurotransmitter.

#### **1.3.1. Modulation of neurotransmitter release**

Evidence that neurotransmitter release is modulated by presynaptic receptors was first shown in vertebrate preparations (see Starke, 1981). Adrenaline and noradrenaline were reported to increase the end plate potentials recorded from rat skeletal muscle (Krnjevic and Miledi, 1958), indicating presynaptic adrenergic receptors were involved in facilitating ACh release from the cholinergic motoneuron terminals. Since then, the release of all types of neurotransmitters has been shown to be modulated by



a whole variety of receptors for different neuromodulators (see Starke, 1981; Watson, 1992a).

Receptors activated by the neurotransmitter released from the presynaptic terminals of the neuron on which they are located are known as autoreceptors. Evidence for this type of self-regulatory receptor was first revealed in 1971 (Johnston and Mitchell, 1971; Polak, 1971) but has since been found in countless vertebrate (Dixon et al., 1979; Michaelson et al., 1979; Lovinger et al., 1993; Vannucchi and Pepeu, 1995) and invertebrate preparations (Breer and Knipper, 1984; Hue et al., 1989; Knipper and Breer, 1989; Trimmer and Weeks, 1989; Parnas et al., 1994; Leitch and Pitman, 1995; Parker and Newland, 1995). The majority of autoreceptors down-regulate transmitter release (Breer and Knipper, 1984; Hue et al., 1989; Knipper and Breer, 1989; Trimmer and Weeks, 1989; Lovinger et al., 1993; Parnas et al., 1994; Leitch and Pitman, 1995; Parker and Newland, 1995; Vannucchi and Pepeu, 1995) although some adrenergic receptors appear to facilitate transmitter release (Dixon et al., 1979).

Presynaptic terminals modulated by chemicals other than their own possess heteroreceptors. Heteroreceptors may be involved in facilitating (Brunelli et al., 1976; Breen and Atwood, 1983; Day and Fibiger, 1993; Kayadjanian et al., 1994) or inhibiting (Baux et al., 1993; Chang and Cheng, 1993; Bellingham and Berger, 1996; Kurokawa et al., 1996) neurotransmitter release.

The effects the neuromodulators have on neurotransmitter release depend on the type of receptor and the intracellular events which they stimulate. As with postsynaptic

receptors, presynaptic receptors may be ionotropic or metabotropic. Activation of ionotropic receptors such as GABA receptors on the terminals of a presynaptic neuron increases the  $\text{Cl}^-$  conductance, resulting in either depolarisations (Burrows and Laurent, 1993; Cattaert et al., 1994) or hyperpolarisations (Hue and Callec, 1983), which may affect voltage-sensitive  $\text{Ca}^{2+}$  channels and ultimately lead to a change in neurotransmitter release.

Activation of presynaptic metabotropic receptors that are coupled to G-proteins evoke second messenger cascades that modulate neurotransmitter release (Baux et al., 1992, 1993; Allgaier et al., 1993). The majority of second messengers act indirectly through protein kinases (Llinás et al., 1985; Nichols et al., 1990; Oka et al., 1996; Somogyi et al., 1996) to modulate ion channels on the presynaptic terminal. For example, activation of 5HT receptors on the presynaptic terminals of the *Aplysia* siphon sensory neurons, causes an increase in the cAMP levels via the enzyme adenylate cyclase (Brunelli et al., 1976), which activates protein kinases (Castellucci et al., 1980) to phosphorylate a specific S (serotonin)-type  $\text{K}^+$  channel (Klein et al., 1982; Siegelbaum et al., 1982; Abrams et al., 1984). Closure of the S-type  $\text{K}^+$  channel by the cAMP-dependent protein kinases (Shuster et al., 1985), prolongs the action potentials in the sensory neurons (Goldsmith and Abrams, 1992) and leads to an increase in  $\text{Ca}^{2+}$  influx (Klein and Kandel, 1980), which enhances transmitter release and thus chemical synaptic transmission.

### **1.3.2. Modulation of the postsynaptic response to neurotransmitters**

Chemical synaptic transmission may also be modulated by neuromodulators activating postsynaptic receptors to modify the response of the postsynaptic neuron to the neurotransmitter released from the presynaptic neuron.

The neuromodulators may modulate ion channels in the membrane of the postsynaptic neuron either directly or indirectly via second messenger pathways to alter the excitability of the postsynaptic neuron and thus the response of the cell to neurotransmitters. For example, activation of postsynaptic muscarinic receptors on insect neurons reduces a  $K^+$  conductance, evoking a slow depolarisation, which increases the excitability of the neurons and the probability that the neurons will evoke spikes in response to sensory afferent stimulation (Benson, 1992; Le Corrone and Hue, 1993; Trimmer and Weeks, 1993).

Alternatively the neuromodulators may activate second messengers pathways via metabotropic receptors, that specifically modulate the receptor activated by the neurotransmitter. Receptors for numerous neurotransmitters have been shown to be modulated by second messengers. Ionotropic receptors such as glutamate receptors (Greengard et al., 1991) and nicotinic cholinergic receptors (Huganir and Greengard, 1990; Swope et al., 1995) as well as metabotropic receptors such as muscarinic cholinergic receptors (Haga et al., 1996), are phosphorylated by protein kinases activated by cAMP. However, not all second messengers pathways involve cAMP. There is evidence that GABA and nicotinic cholinergic receptors on the cockroach

motoneuron  $D_f$  are modulated by muscarinic receptors via a phosphoinositol pathway, which causes a rise in intracellular  $Ca^{2+}$  levels (David and Pitman, 1996c).

The studies described above, reveal the main mechanisms underlying the modulation of chemical synaptic transmission. However, the finer details of some of the stages (e.g. the synaptic vesicle release machinery) are as yet unknown.

#### **1.4. The locust forewing stretch receptor/first basalar motoneuron synapse**

Much of the evidence, which forms the basis of our understanding of the modulation of synaptic transmission, has been pieced together from studies on different synapses and different species. Although forming a universal picture of this process is very important, using different synapses often gives conflicting results. Using one preparation to study all aspects of modulation at a particular synapse would provide an insight as to how different neuromodulators interact to modulate transmission between two identified neurons

Due to the large number and small size of the neurons in vertebrate preparations, studying synaptic transmission between two individual identified neurons would prove difficult. Individual neurons from invertebrate preparations however, are larger and are easier to identify. In addition, only one or a small number of neurons are involved in controlling certain behaviours rather than whole populations of neurons, as is the case in the vertebrate central nervous system (CNS). Therefore, the connections between large identified invertebrate neurons are attractive models to study synaptic modulation.

The monosynaptic connection between the locust forewing stretch receptor (fSR) and first basalar motoneuron (BA1) is one such model. The fSR is a single sensory neuron attached to hinge of each forewing in the locust (Gettrup, 1962). There are therefore 2 fSRs and also 2 hindwing SRs (hSRs). The cell body of each SR is suspended in an elastic strand between an internal cuticular wall in the thorax and a sclerite of the wing hinge (Gettrup, 1962). The cell body gives rise to a large axon which runs into nerve (N) 1D<sub>2</sub> (Campbell, 1961). The diameter of the SR axon in N1D<sub>2</sub> is relatively large (6µm) compared to the diameter of other axons in this nerve (2.5-4 µm), which come from the multicellular scolopidial organ also associated with the wing hinge. The SR axons project towards the CNS via N1D<sub>2</sub> but before they reach the CNS they bifurcate sending one branch into the prothoracic ganglion via N6 and one branch into the mesothoracic ganglion via N1 (Altman and Tyrer, 1977). No other sensory neuron axons bifurcate in this way (Burrows, 1975). Having entered the CNS, each SR neuron forms extensive arborizations which are ipsilateral and mainly in the dorsal neuropil (Altman and Tyrer, 1977).

Recordings made from the nerves that contain the SR axons (N1D<sub>2</sub>, Gettrup, 1962; N1, Burrows, 1975) reveal that spikes with a large amplitude, which are thought to originate from the SR, are evoked when the wing is elevated. Spikes with a smaller amplitude are also evoked, but these are thought to originate from the scolopidial organ (Gettrup, 1962). The frequency of spikes recorded from the fSR axon depends on the positional angle of the wing (Gettrup, 1962; Burrows, 1975). If the wing is held at 90°, the fSR fires, 15-25 times per second and if it is held at 130° it fires at 30-40 times per second. It also depends on the rate at which the wing is moving

upwards. When the wing is being elevated the frequency of spikes increases, up to 300 times per second, but if the movement is stopped and the wing is held elevated, the frequency decreases to a static one, which depends upon the positional angle of the wing (Gettrup, 1962; Gray and Robertson, 1994). The SR therefore provides information on the angle of the wing and its movement. The frequency and number of spikes recorded from the fSR axon also depends on the maturation of the locust (Gray and Robertson, 1994), the phase it is stimulated in relation to wing cycle and motoneuron activity (Möhl, 1985), and the yaw movements of the locust (Möhl, 1985). The spikes in the SR axon are conducted at a velocity of 2.5 m/s in immature locusts and 3.6 m/s in adult locusts, meaning it will take a spike 3.6 ms to travel from the wing to the CNS in a adult locust with a SR axon 13 mm long (Gray and Robertson, 1994).

Within the CNS, each SR makes excitatory connections with ipsilateral wing depressor motoneurons (Burrows, 1975, Reye and Pearson, 1987; Pearson and Ramirez, 1990). The study by Burrows (1975) on the locust *Schistocerca gregaria* revealed that the fSRs connect with the dorsal longitudinal depressor motoneurons in the pro- and mesothoracic ganglia, BA1 in the meso- and metathoracic ganglia and the second basalar and subular motoneurons in the mesothoracic ganglia. Stimulation of the SRs evokes depolarising responses in the depressor motoneurons with a constant latency of 1 - 2.5 ms, which indicates the connections between the SRs and the depressor motoneurons are monosynaptic (Burrows, 1975). The SRs also make inhibitory connections with ipsilateral elevator motoneurons, including the first and second tergosternal and the anterior and first posterior tergocoxal elevator motoneurons (Burrows, 1975). Stimulation of the SRs evokes inhibitory postsynaptic

potentials (IPSPs) in the elevator motoneurons with a constant latency of 4-6 ms, indicating that an inhibitory interneuron is interposed between the SRs and elevator motoneurons (Burrows, 1975). Similar results were reported in a study by Reye and Pearson (1987) in the locust *Locusta migratoria*, although no IPSPs were recorded from the elevator motoneurons. Burrows (1996) suggested this could be explained by the difference in the gain between the elevator motoneurons and the interneurons which also receive inputs from the SRs.

Based on what is known about the connections the SRs make with wing motoneurons, it would appear that they are part of a simple sensory feed-back loop. Elevation of the wing initiates spikes in the SRs, which excite depressor motoneurons causing the depressor muscles to contract and bring the wing back down. Stimulation of the SRs also inhibits elevator motoneurons, which would stop the elevator muscles contracting and prevent the wing from being elevated further. When the wing is brought back down the SRs stop firing, so they stop exciting the depressor motoneurons and prevent the wing from being depressed further. There are other wing proprioceptors e.g. the tegula which are activated when the wing is depressed and excite elevator motoneurons to raise the wing (Burrows, 1976).

The SRs also play an important role in regulating the flight central pattern generator (CPG). Evidence of this was first reported by Gettrup (1962). Destroying the SRs by local cauterisation caused the stroke frequency of a flying locust to decrease significantly, sometimes to less than half of the original frequency. This indicated that the SRs are involved in a sensory feedback system that increases the frequency of the flight rhythm (Wilson and Gettrup, 1963). This was confirmed by further studies

(Pearson et al., 1983; Reye and Pearson, 1988), which also revealed that the fSR could reset and entrain the flight rhythm when stimulated at an appropriate phase in the wing beat cycle (i.e. close to onset of depressor activity).

As the fSR is important in the regulation of the flight motor pattern, modulating the fSR and its synaptic connections would provide an efficient way of modifying sensory information before it is integrated into the flight motor pattern. Therefore, it is highly probable that the synapse between the fSR and BA1 is modulated. A recent study by Leitch and Pitman (1995), has already revealed that presynaptic muscarinic cholinergic receptors are involved in down-regulating ACh release from the fSR terminals. As GABA (Robertson and Pearson, 1983; Pearson and Robertson, 1987; Reye and Pearson, 1987; Robertson and Wisniowski, 1988), glutamate (Bicker et al., 1988; Dubas, 1990, 1991), octopamine (Orchard et al., 1993), 5HT and dopamine (Claassen and Kammer, 1986) are all important in the modulation of insect flight it is possible that the fSR/BA1 synapse is also modulated by other neuromodulators.

### **1.5. Aims of this study**

The main aim of this study was to examine the possibility that cholinergic synaptic transmission between two identified neurons is modulated by a variety of neuromodulators. Electrophysiology, neuropharmacology, electron microscopical (EM) and confocal immunocytochemistry (ICC) were used to confirm that presynaptic muscarinic receptors are involved in the modulation of the fSR/BA1 synapse in the locust *Locusta migratoria* and to identify other neuromodulators and their mechanisms of action that modulate the fSR/BA1 synapse.



## **Chapter 2. General materials and methods.**

### **2.1. Animals**

Adult male and female *Locusta migratoria* L. phase gregaria were used in all experiments in this study. The locusts were kept at a population density (50-70 per cage) high enough to prevent them reverting to a solitary phase. The cages were kept in an insectary that had a light/dark cycle of 12L:12D. The temperature in the insectary was 30°C and the humidity was 50%. The locusts were provided with fresh grass daily.

### **2.2. Electrophysiological methods**

#### **2.2.1. The dissection**

The locust was killed by decapitation and the wings and legs removed with scissors. The body of the locust was positioned ventral side upper-most on a sylgard (Sylgard 184, Dow Corning, Wiesbaden, Germany) based petri dish and pinned through the anterior dorsal cuticle and the posterior end of the abdomen. The petri dish was positioned beneath a Zeiss dissecting microscope. A shallow incision was made with a scalpel along the lateral and posterior edges of the thoracic cuticular plate between the first and third pairs of legs so that the thoracic plate could be removed carefully. The exposed thoracic cavity was perfused with locust saline (Table 2.1) and the connective tissue and air sacs were gently removed to expose the thoracic nerve cord (pro-, meso- and metathoracic ganglia, Figure 2.1). Mesothoracic nerve 1 (N1) and prothoracic nerve 6 (N6) on either side of the nerve cord were identified before cutting mesothoracic

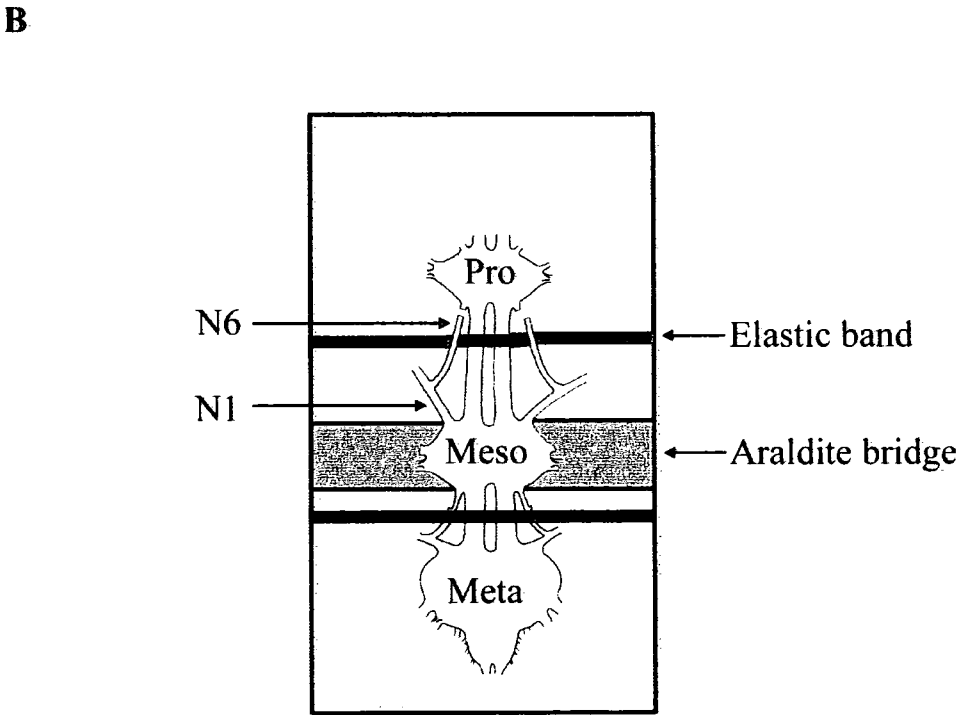
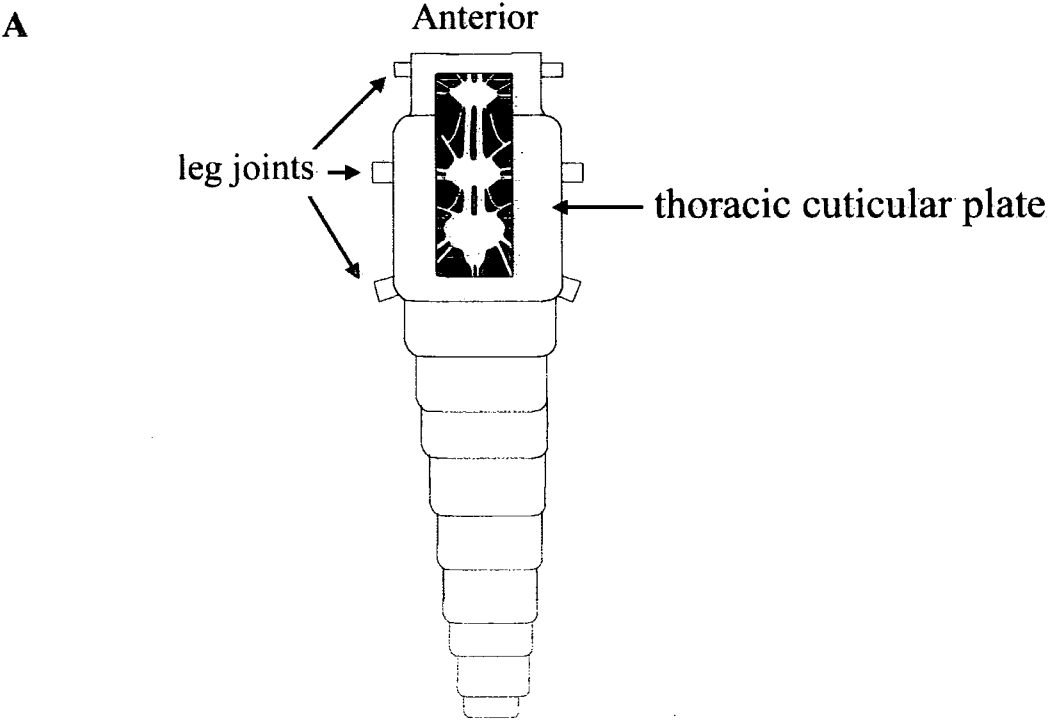
**Table 2.1. Locust saline used in this study.**

To make 5 litres.

Stock solutions	Quantity	Final saline molarity (mM)
2M NaCl	350 ml	140
1M KCl	25 ml	5
1 M MgCl <sub>2</sub>	5 ml	1
1 M CaCl <sub>2</sub>	25 ml	5
0.5 M NaHCO <sub>3</sub>	40 ml	4
HEPES	7.51g	6.3
Deionised distilled water	4555ml	

**Figure 2.1. The locust thoracic nerve cord.** (A) A diagram showing the exposed nerve cord in the locust thoracic cavity. The locust is positioned ventral side-up on a petri dish and a window of cuticle is cut from the thoracic cuticular plate between the first and third pairs of legs to reveal the pro-, meso- and metathoracic ganglia. Mesothoracic nerve 1 (N1) is cut distal to the junction with prothoracic nerve 6 (N6) and prothoracic N6 is cut at a point just distal to the prothoracic ganglion. (B) The isolated locust thoracic nerve cord secured to a plastic tile. The mesothoracic ganglion is positioned over an araldite bridge and the prothoracic/mesothoracic and mesothoracic/metathoracic connectives are secured underneath elastic bands.

**Figure 2.1. The locust thoracic nerve cord.**



nerve 1 distal to the junction with prothoracic N6. Prothoracic N6 was cut just distal to the point N6 joins the ganglion so a substantial length of prothoracic N6 was left attached to mesothoracic N1. The other nerves innervating the thoracic ganglia were cut so the thoracic nerve cord could be carefully removed from the locust.

The isolated thoracic nerve cord was then placed ventral side uppermost (prothoracic ganglion to the top) on a small black plastic tile (16 x 9 x 1 mm) (Figure 2.1B). The mesothoracic ganglion was positioned on top of a lateral araldite hump and the prothoracic/mesothoracic and mesothoracic/metathoracic connectives were secured underneath elastic bands. The sheath over the mesothoracic ganglion was carefully removed with sharpened watch makers forceps to expose the cell bodies on the ventral surface. The preparation was transferred to an experimental chamber containing 2 mls of circulating oxygenated saline (Figure 2.2A). All experiments were carried out at room temperature (19-23°C).

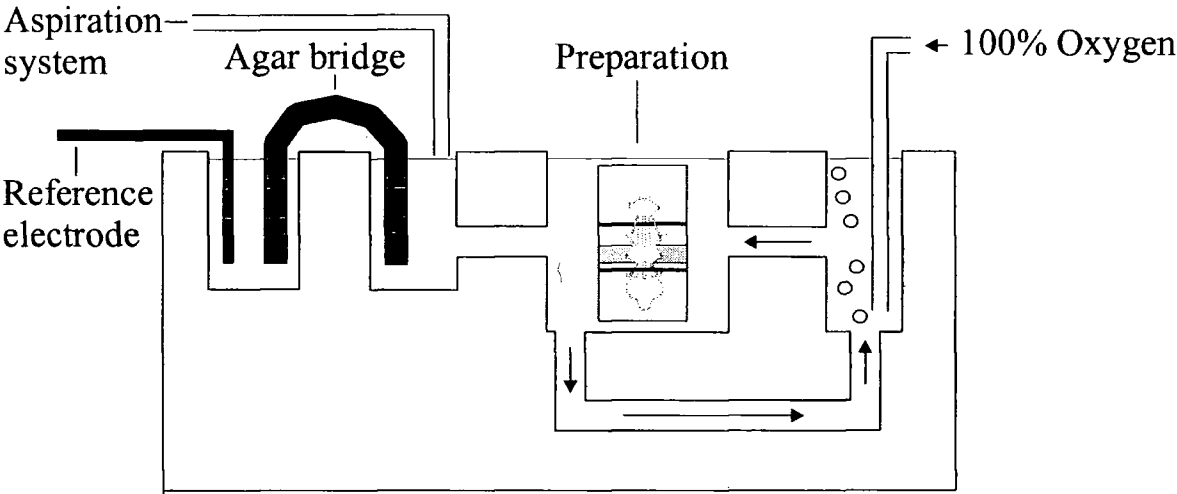
### **2.2.2. The experimental chamber**

The experimental chamber consisted of three wells connected by channels cut into a perspex block (Figure 2.2A). The middle well was cut so that the preparation could be laid in it at a 45° angle and viewed through a perspex panel on the side of the block. A tube connected to a gas cylinder containing 100% oxygen was inserted into the right well and fixed so its end was positioned near the bottom of the well. Due to the arrangement of the channels between the right and middle well, oxygen bubbles released from the tube in the right well, drew the saline around the channels and over the preparation in the middle well. The circulating saline maintained neuronal resting

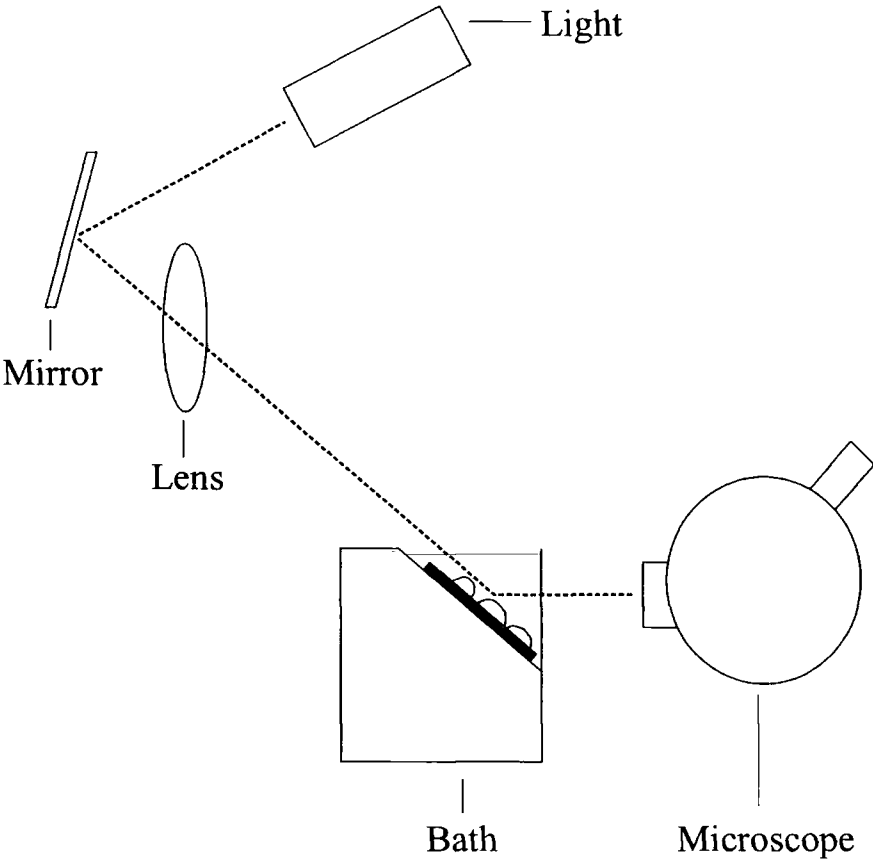
**Figure 2.2. The experimental chamber used for electrophysiological recordings.** (A) A front view of the bath. A tube inserted into the front right well releases 100% oxygen bubbles which draw the saline through the channels and around the interconnected wells. This ensures that the preparation placed in the middle well is perfused with recirculating saline. Test compounds and saline are added manually to the right well to ensure they are circulated. Excess saline is removed from the bath via an aspiration system positioned over the left well. An agar bridge connects the right well with a separate fourth well containing the reference electrode which allows the recording electrode (not shown) and the reference electrode to be electrically connected. (B) A transected side view of the bath showing the preparation. A binocular microscope is suspended in front of the bath so the preparation can be viewed. The nerve cord is illuminated by an indirect light source to avoid heating the preparation. Light shone from a dissecting microscope light source is reflected off a mirror and through a convex lens onto the preparation.

**Figure 2.2. The experimental chamber used for electrophysiological recordings.**

**A**



**B**



potential values and synaptic transmission over a period of hours. Test compounds and drug-free saline were added to the right well so they would be circulated with the saline. The left well was connected to the middle well by a single channel. A suction tube positioned over the left well, removed any excess saline from the bath. The left well was connected by an agar bridge to a separate fourth well containing a silver/silver chloride (Ag/AgCl) reference electrode. This arrangement allowed the recording electrode and the reference electrode to be electrically connected without the reference electrode being in the experimental chamber (hence isolating it from fluctuating saline levels).

The bath was fastened securely to a solid wooden block which was positioned on a MICRO-g vibration isolation table (Technical Manufacturing Corporation, Peabody, U.S.A.) and inside a Faraday cage to reduce electrical noise. The nerve cord could be viewed with a suspended binocular microscope positioned in front of the bath and was illuminated with an indirect light source to prevent the light heating the bath (Figure 2.2B). Light from a dissecting microscope light source was reflected off a mirror attached to the back of the Faraday cage, through a convex lens and forward onto the nerve cord. This allowed the cell body of the motoneuron BA1 to be visualised. The cell body of BA1 could be identified on the basis of its position in relation to its neighbours. It is the most medial and caudal cell body in the anterior group of large cell bodies on the ventral surface of the mesothoracic ganglion. The identity of the motoneuron was confirmed by reimpaling the cell with an electrode containing 4% hexamminecobalt(III) chloride following an experiment. The hexamminecobalt chloride was injected into the motoneuron and the ganglion processed for histology and Timms silver intensification (2.3.2).



### 2.2.3. Impalement of BA1

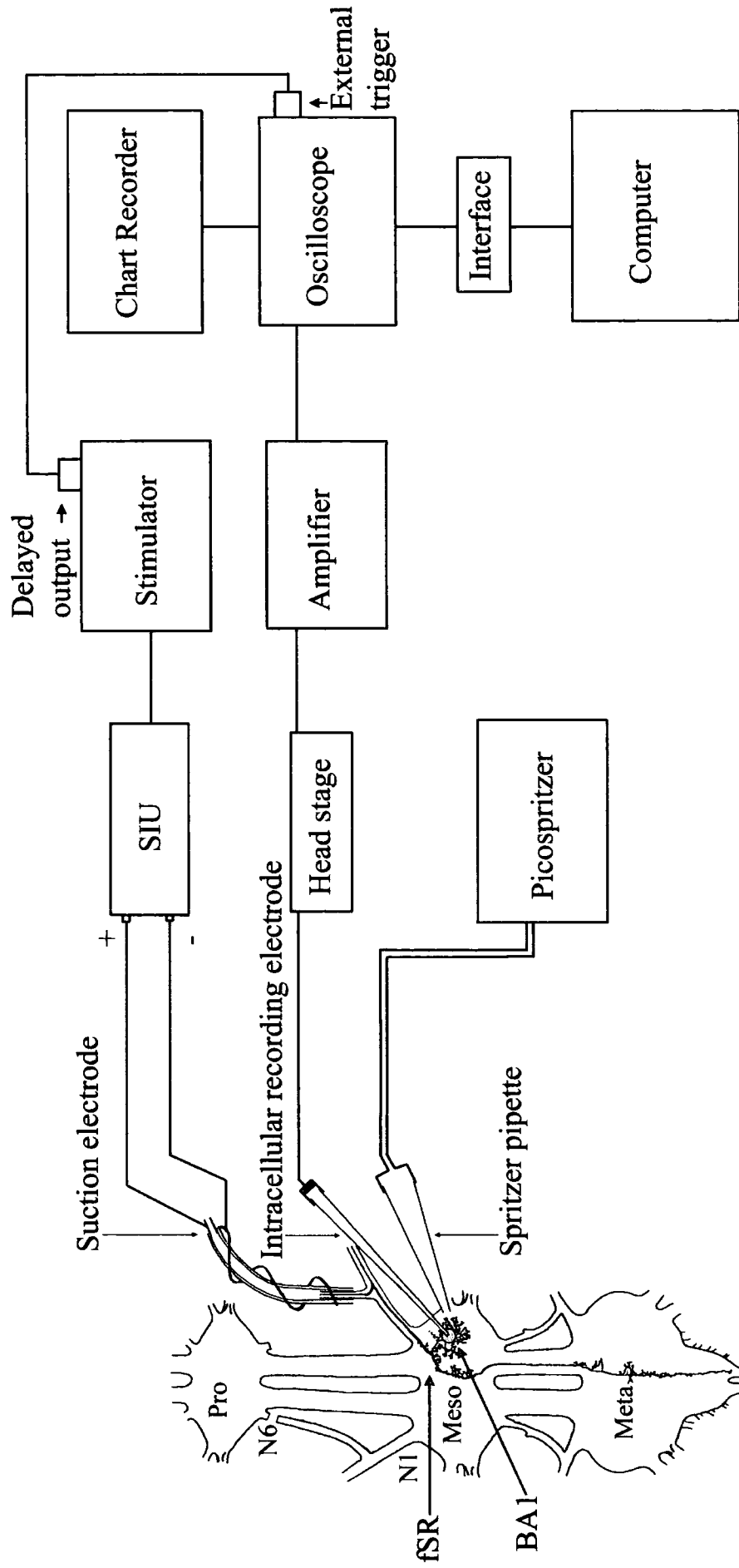
BA1 was impaled with a single glass microelectrode back-filled with 2M potassium acetate (Figure 2.3). Microelectrodes with a resistance of 10-20 M $\Omega$  were made by pulling a borosilicate glass capillary tube with integral filament (World Precision Instruments, Stevenage, U.K.) on an Ensor moving-coil microelectrode puller (Clark Electromedical, Reading, U.K.). The microelectrode was inserted into a holder, containing a silver/silver chloride pellet, which was attached to a head stage of an Intra 767 electrometer amplifier with a gain of  $\times 10$  (World Precision Instruments, Stevenage, U.K.). The amplifier was connected to a (DSO) 1604 oscilloscope equipped with a 260 wave form processor (Gould Instrument Systems, Ilford, U.K.). The recording and stimulating circuit is shown in Figure 2.3.

Having advanced the recording microelectrode into the experimental chamber using a manual micromanipulator (World Precision Instruments, Stevenage, U.K.), the amplifier was switched on. The potential difference between the recording microelectrode and the Ag/AgCl reference electrode, which was connected to earth via the amplifier, was displayed digitally and set to zero using an offset control on the amplifier. The microelectrode resistance was then measured by injecting 1 nA of current through the active probe. From Ohm's law, the voltage deflection evoked by 1 nA of current was proportional to the resistance of the electrode (1mV/M $\Omega$ ).

The microelectrode was advanced towards to cell body of BA1 under visual control. When the tip of the electrode touched the surface of BA1 the electrode was 'tickled'

**Figure 2.3. The equipment used to evoke and record EPSPs and acetylcholine responses from BA1.** The electrical stimulation of the fSR axon evokes excitatory postsynaptic potentials (EPSPs) in BA1. Due to the fSRs unique bifurcation electrical stimulation of prothoracic N6 via a suction electrode does not stimulate any other sensory neurons in mesothoracic N1 except the fSR. The suction electrode is connected to a stimulator via a stimulus isolation unit. A delayed output from the stimulator is connected to the external input trigger on the oscilloscope. The EPSPs are recorded from BA1 with a intracellular recording microelectrode. The microelectrode is connected to an amplifier via a head stage. The amplifier is connected to an oscilloscope and a personal computer (via a data translation interface) equipped with a synaptic current analysis program so that the EPSPs can be displayed and recorded. Acetylcholine (ACh) responses were evoked in BA1 by locally-applying ACh the cell body of BA1. Brief pulses of ACh were pressure-applied from a 'spritzer' pipette attached to a picospritzer. The resulting ACh responses were recorded on a chart recorder attached to the oscilloscope.

**Figure 2.3. The equipment used to evoke and record EPSPs and acetylcholine responses from BA1.**



with a brief overcompensation of the negative capacitance feedback, which allowed the microelectrode to penetrate the cell. Cell penetration was signalled by the rapid appearance of the resting potential (-50 to -70 mV). Having impaled the cell, the bridge in the amplifier was balanced to remove the portion of the recorded voltage caused by current passing through the microelectrode. The bridge was balanced by injecting square pulses (10 mV, 5 ms) into BA1 and removing the transient spikes at the start and end of the pulses, displayed on the oscilloscope, by adjusting the bridge circuit control on the amplifier.

The input resistance of BA1 was measured after the bridge had been balanced. Brief (500 ms) pulses of hyperpolarising current (I) were injected into BA1 via the active probe at a rate of 1 Hz which caused voltage deflections in the membrane potential (V), which were measured. The input resistance was then calculated using Ohms law ( $R=V/I$ ).

#### **2.2.4. Stimulation of the fSR**

Synaptic transmission between the sensory afferent fSR and the motoneuron BA1 was evoked by electrically-stimulating the fSR axon. The fSR could be selectively stimulated due to its unique branching pattern (Figure 2.3). As the fSR axon comes from the periphery towards the CNS in nerve 1D<sub>2</sub> (Campbell, 1961) it bifurcates, sending one branch into mesothoracic N1 and one branch into prothoracic N6. As no other sensory neurons bifurcate into these two branches (Burrows, 1975), electrical stimulation of prothoracic N6 does not stimulate any other sensory neurons in mesothoracic N1, except the fSR.

Prothoracic N6 containing the fSR axon was drawn into a suction electrode, with the aid of a syringe attached to the suction electrode, ensuring a snug fit and thereby minimising current leakage. The suction electrode was connected to a S48 square pulse stimulator equipped with a SIU5 stimulus isolation unit (Grass Instrument Division, West Warwick, U.S.A (Figure 2.3), which allowed brief single or repetitive stimuli to be applied to the fSR via the suction electrode. The stimulator was also connected to the external trigger of the oscilloscope (Figure 2.3), so an oscilloscope sweep was started for observation when the fSR was stimulated. The horizontal position of the response on the oscilloscope screen could be altered for convenience by delaying the stimulator trigger.

Electrical antidromic stimulation of prothoracic N6 with brief depolarising voltage pulses (500  $\mu$ s), evoked excitatory postsynaptic potentials (EPSPs) in BA1. The voltage needed to evoke EPSPs in BA1 ranged from 0.2- 6V. There was no relation between the voltage used to stimulate the fSR and the amplitude of EPSPs recorded from BA1 i.e. increasing the voltage did not increase the amplitude of the EPSPs. Altering the frequency of stimulation however, did affect the amplitude of the EPSPs recorded from BA1. An increase in the frequency ( $> 0.5$  Hz) of stimulation caused the amplitude of the EPSPs to decrease. Therefore, the fSR was stimulated at a set frequency (0.1 Hz) to avoid any frequency-induced variation in the EPSP amplitude during experiments.

Individual EPSPs were displayed and recorded on the Gould (DSO) 1604 oscilloscope and simultaneously on the screen of a personal computer equipped with a synaptic

current analysis program (Strathclyde Electrophysiological Software, John Dempster, University of Strathclyde, U.K.). A Data Translation DT2812 interface (Data Translation, Marlboro, U.S.A.) interposed between the oscilloscope and computer (Figure 2.3) allowed the analogue voltage signals from the oscilloscope to be converted to a digital form so they could be collected by the synaptic current analysis program and stored for later analysis.

In all experiments, groups of 8 successive (0.1Hz) electrically-evoked EPSPs were recorded from BA1 at 5 minute intervals and averaged using the Gould (DSO) 1604 oscilloscope equipped with a 260 wave form processor (Gould Instrument Systems, Ilford, U.K.). Analysis of the individual EPSPs, revealed that there was very little variation between the amplitudes of successive EPSPs recorded in each group. The individual EPSP amplitudes in each group only varied by  $0.5 \pm 0.07$  mV (mean  $\pm$  SEM;  $n = 20$ ) in a sample of 20 experiments.

Experiments were started when the EPSP amplitude was regarded as stable i.e. when the averaged amplitudes of at least three groups of 8 successive EPSPs were the same. This occurred approximately 40 minutes after impalement. The mean amplitude of control EPSPs recorded from BA1 was  $4.4 \pm 0.2$  mV (mean  $\pm$  SEM;  $n = 128$ ) and the mean duration was  $114.7 \pm 4.1$  ms (mean  $\pm$  SEM;  $n = 128$ ).

#### **2.2.5. Experimental protocol**

After the control EPSP amplitude recorded from BA1 had stabilised, test compounds dissolved in saline solution were added to the front left well of the experimental

chamber in 20  $\mu$ l aliquots. Care was taken not to disturb the intracellular electrode when compounds were added to the experimental chamber. As the volume of saline in the experimental chamber was 2 ml, this resulted in a 100 fold dilution.

Immediately after adding the test compounds, a group of 8 successive EPSPs were recorded to determine if the physical activity of adding a compound to the bath had disturbed the intracellular electrode. After this, groups of 8 successive electrically-evoked EPSPs were recorded from BA1 at 5 minute intervals as described previously. The membrane potential and input resistance of BA1 were also monitored to assess if the test compounds had an effect on the membrane conductance of BA1 (see 2.2.3). A two-tailed t-test was used to determine if the input resistance of BA1 was significantly different in the absence (control) and presence of the compounds.

After any drug-related changes in the amplitude or duration of the EPSPs were recorded, the test compounds were washed out of the experimental chamber by flushing the preparation with fresh saline. Fresh saline was added carefully to the right well, so as not to disturb the impalement, and the excess saline and test compounds were removed from the bath by the aspiration system positioned over the left well. EPSPs were recorded as described previously to demonstrate recovery after washing. If it was possible, washing was continued until the amplitude and duration of the EPSPs returned to control levels.

The effects of the test compounds on the EPSP amplitude and duration were calculated as percentage changes in the control EPSP amplitude and duration. As percentage values may not be normally distributed, the data was normalised using an arc-sine transformation ( $\text{ASIN}[\text{SQRT}[/100]]$ ). Means and standard errors of means were

calculated from the arc-sine transformed values before back-transforming. All percentage means and standard errors of percentage means reported in this study have been arc-sine transformed unless otherwise stated. A two-tailed paired t-test was used to assess the significance of the percentage changes between control EPSP amplitudes and EPSP amplitudes in the presence of compounds. A two-tailed unpaired t-test was used to assess the significance of the percentage changes in EPSP amplitudes between different experiments.

#### **2.2.6. Pressure-application of ACh to the cell body of BA1**

To examine the effects of test compounds on the responses of BA1 to ACh, ACh was locally-applied to the cell body of BA1. A pipette made from pulling a soda lime glass microhaematocrit tube (Kimble, Vineland, U.S.A.) on a PB-7 two-stage electrode puller (Narishige, Tokyo, Japan) was back-filled with ACh (100 mM). The pipette was attached via a holder to a Picospritzer II (General Valve Corporation, New Jersey, U.S.A.) and positioned using a manual micromanipulator so the tip was in close proximity to the cell body of BA1 (Figure 2.3).

Pulses of ACh pressure ejected from the tip of the pipette evoked depolarising responses in BA1. Brief pulses (100-500 ms) of ACh were applied at 10-20 psi and at a rate of 0.01 Hz. Fine adjustments of the pulse length and pressure and the distance of the tip of the pipette from BA1 prevented BA1 from becoming desensitised to ACh and allowed successive depolarising responses of constant amplitude to be evoked. The mean amplitude of control ACh responses recorded from BA1 was  $18.7 \pm 0.9$  mV (mean  $\pm$  SEM;  $n = 42$ ). A Kipp and Zonen chart recorder (Philip Harris



Scientific, Blyth, U.K.) connected to the oscilloscope recorded the ACh responses.

To examine the effects of the test compounds on ACh responses the compounds were applied and the effects on the ACh responses calculated as previously described (2.2.5.).

### **2.3. Staining the fSR for light microscopy.**

#### **2.3.1. Back-filling the fSR *in vitro*.**

The thoracic nerve cord was removed from the locust as previously described (2.2.1.) and placed on the an upturned petri dish. Due to the unique bifurcation of the fSR axon as it approaches the CNS, the fSR in the mesothoracic ganglion was selectively filled via prothoracic N6. Prothoracic N6 was placed in a Vaseline well, which was filled with a 100 mM solution of hexaminecobaltic chloride. The well was covered with Vaseline and then the petri dish was placed in a moist chamber for 3 days at 4°C to allow the cobalt chloride to diffuse through the fSR axon.

#### **2.3.2. Histology.**

The Vaseline and cobalt chloride were removed from prothoracic N6. The nerve cord was then immersed for 15 minutes in ammonium sulphide, diluted to 5% with locust saline, to allow the cobalt ions in the fSR to precipitate as cobalt sulphide (Pitman et al., 1972). The nerve cord was washed in locust saline and fixed for 30-60 minutes at room temperature in 5% formaldehyde (BDH, Poole, U.K.), which had been diluted

with locust saline and buffered to pH 7.2. It was then dehydrated in an ethanol series (50%-70%) and left overnight in 70% ethanol at room temperature.

The following day the thoracic nerve cord was processed using Timms silver-intensification method (Bacon and Altman, 1977; Brogan and Pitman, 1981). The intensification was carried out in the dark as the silver nitrate is photosensitive. Also the nerve cord did not come into contact with any metal in order to prevent the precipitation of metal ions on the surface of the nerve cord. The nerve cord was rehydrated in 50 % ethanol and then washed in deionised distilled water (DDW). The nerve cord was then immersed in DDW in an uncovered glass vial, which was placed in an oven set at 60°C for 15-30 minutes. It was then transferred to a glass vial containing Timms staining solution (Appendix 1) already warmed to 60°C and left in the oven for 1-2 hours. A few drops of a 1% solution of silver nitrate were added to the glass vial containing the nerve cord which turned the Timms slightly white. If too much silver nitrate was added the solution became cloudy but it could be diluted with more Timms (60°C). The vial was replaced in the oven at 60°C but checked at 20 minute intervals to prevent silver collecting on the surface. If the cobalt had not intensified after an hour, the nerve cord was placed in a vial with fresh Timms (60°C) and the silver nitrate was added as before. The vial was replaced in the oven and checked every 20 minutes.

After the cobalt in the fSR had intensified, the nerve cord was placed in fresh Timms (60°C) for 5 minutes before placing it in DDW for 10-15 minutes at room temperature. Nerve cords that were to be viewed as wholemounts were dehydrated in a graded ethanol series (50% - 100%). It was essential the nerve cord was completely

dehydrated in 3 x 100% ethanol before clearing the tissue in methyl salicylate. The nerve cords were then mounted in DPX mounting medium (Agar Scientific, Stansted, U.K.) on cavity slides. The projections of the fSR in the meso- and metathoracic ganglia were drawn from the wholemounts with the aid of a drawing tube fixed to a Optiphot compound microscope (Nikon U.K. Ltd, Surrey, U.K.).

Nerve cords for serial sectioning were dehydrated in an ethanol series (50% -100 %) before infiltrating with a 100% ethanol/propylene oxide solution. The nerve cords were transferred to pure propylene oxide before immersing them in a propylene oxide/soft araldite (Appendix 2) in an uncovered glass vial. The vial was placed in an oven set at 45°C for 30 minutes to allow the propylene oxide to evaporate. The nerve cords were then immersed in a vial of fresh soft araldite and replaced in the oven for 30 minutes. Individual ganglia were positioned in moulds and then covered in fresh soft araldite before polymerisation in an oven set at 60°C for 48 hours.

The mesothoracic ganglia were cut into transverse serial sections (5 µm) with a glass knife mounted on a Reichert-Ultracut ultratome (Leica Cambridge Ltd, Cambridge, U.K.). The sections were mounted on glass slides and the fSR processes within the sections were drawn with the aid of a drawing tube fixed to a Optiphot compound microscope (Nikon U.K. Ltd, Surrey, U.K.). The level at which each slice had been taken through the mesothoracic ganglia was identified using anatomical guides (Tyrer and Gregory, 1982; Pflüger et al., 1988).

## **2.4. Staining the fSR for electron microscopy**

### **2.4.1. Back-filling the fSR *in vivo***

To preserve the ultrastructure of the tissue in the mesothoracic ganglia for electron microscopy (EM), the fSR was back-filled *in vivo*. The wings of the locust were removed with scissors and then the locust was positioned ventral side upper-most on a sylgard-based petri dish. It was secured by pinning its legs to the Sylgard and the dish was positioned beneath a Zeiss dissecting microscope. A small window of cuticle was cut from the thoracic plate, anterior to the 2nd pair of legs so that the mesothoracic ganglion remained covered by cuticle. The thoracic cavity was perfused with locust saline and then the connective tissue and air sacs were gently eased to the side to expose mesothoracic N1 and prothoracic N6 on one side of the nerve cord. Prothoracic N6 was cut just distal to the point N6 joins the ganglion so a substantial length of prothoracic N6 was left attached to mesothoracic N1. A parafilm shield was placed over the thoracic cavity and prothoracic N6 was placed on top of this before the sides of the thoracic cavity were sealed with Vaseline. Prothoracic N6 was placed in a Vaseline well made on the parafilm shield before the well was filled with 4% Horseradish peroxidase (HRP) made up in 0.2M Tris buffer (pH 7.4) (Appendix 3) and sealed with Vaseline. Moist chambers containing the locusts were incubated for 4-5 days at 4°C to allow the HRP to diffuse through the fSR.

#### 2.4.2. Electron microscopy

The mesothoracic ganglia containing the HRP-filled fSR were processed for EM according to the method described by Leitch and Laurent (1996). The HRP, Vaseline and parafilm shield were removed and the thoracic cavity was perfused with locust saline. The rest of the thoracic cuticular plate was removed to expose the meso- and metathoracic ganglia before the nerve cord was fixed *in situ* for 5-10 minutes in 2.5% glutaraldehyde (Agar Scientific, Stansted, U.K.) in 0.1M Sorenson's phosphate buffer (pH 7.4) (Appendix 4) containing 0.2M sucrose. The nerve cord was removed from the thorax and transferred to a vial containing fresh fixative. The posterior-side of each mesothoracic ganglion was dried before quickly mounting on a platform with cyanoacrylate glue. The platform was immediately immersed and secured in the tissue chamber of a Vibroslice 725M tissue slicer (Campden Instruments Ltd, U.K.) filled with fresh fixative. A razor blade was secured in the vibrating arm of the tissue slicer and used to cut the ganglia into serial transverse sections (150-200  $\mu\text{m}$  thick).

The tissue slices were fixed for a further 2 hours in fresh fixative before being washed thoroughly in 0.1 M Sorenson's phosphate buffer (pH 7.4) containing 0.2M sucrose, followed by 0.2 M Tris buffer (pH 7.4). They were then placed in 0.5M cobalt chloride in 0.2M Tris buffer for 10 minutes before being washed in 0.2 M Tris buffer (pH 7.4), followed by 0.1 M Sorenson's phosphate buffer (pH 7.4) minus the sucrose. The slices were then transferred to a vial containing 50 mls of incubation medium containing diaminobenzadine (DAB) (Appendix 5) and placed in an oven set at 37°C. After 10-15 minutes 20  $\mu\text{l}$  of glucose oxidase was added to the medium which acted on the  $\beta$ -d-glucose to generate hydrogen peroxidase. The slices were replaced in the oven but were

examined under a dissecting microscope at regular 5 minute intervals to monitor the development of the DAB-HRP reaction product. The reaction was stopped by washing the slices in 0.1 M Sorenson's phosphate buffer (pH 7.4) minus the sucrose and at this time the slices were checked to identify successful fills of the fSR. If the fSR was successfully filled, the slices were incubated in 1% osmium tetroxide (Oxkem Limited, Oxford, U.K.) and then washed thoroughly in DDW. The slices were then stained *en bloc* by incubating them in 2% aqueous uranyl acetate (BDH, Poole, U.K.) for 30 minutes in the dark. After washing in DDW, the slices were dehydrated in an ethanol series (50%-70%) before transferring them for 30 minutes to a 70% ethanol/LR White medium grade resin (Agar Scientific, Stansted, U.K.) solution. The slices were placed in two fresh changes of LR White resin before being infiltrated with it overnight. The next day the slices were placed in a fresh change of LR White resin for 30 minutes before being positioned in a suitable mould and covered in fresh LR White. The tissue was embedded by placing the moulds in an oven set at 50-55°C overnight.

Ultrathin sections for electron microscopy (70 nm) were cut with a glass knife mounted on a Reichert-Ultracut ultratome (Leica Cambridge Ltd, Cambridge, U.K.). The sections were mounted on nickel grids (mesh and slot) which had been coated with Formvar (Agar Scientific, Stansted, U.K.) and carbon-coated in a model 12E6/1288 carbon coater.

To identify the level at which each slice had been taken through the mesothoracic ganglion, semi-thin sections (1  $\mu$ m thick) were also cut from the blocks. These were mounted on glass slides and stained with Toluidine blue before being examined under an Optiphot compound microscope (Nikon U.K. Ltd, Surrey, U.K.). The level at which

each slice had been taken through the ganglion was identified using anatomical guides (Tyrer and Gregory, 1982; Pflüger et al., 1988).

## **2.5. Electron microscopical immunocytochemistry**

Ultra-thin sections for immunocytochemistry were incubated in the solutions needed to immunolabel the section by floating the grids section-side down on droplets of the solutions placed on clean squares of dental model wax. The squares of dental wax were placed in covered petri dishes with moistened filter paper to create a moist environment and prevent the droplets from evaporating. A section was washed by floating the grid for 5 minutes on a droplet of DDW and repeating this 4 times on fresh droplets.

Prior to immunolabelling the sections were first etched for 2-5 minutes with a saturated solution of sodium metaperiodate (Bendayan and Zollinger, 1983). The sections were washed thoroughly and then incubated for 30 minutes with 5% normal goat serum in bovine serum albumin (BSA) /Tris buffer (pH 7.4) (Appendix 6) to block any nonspecific staining. Sections were then incubated for 2-3 hours at room temperature in the primary antibody diluted with BSA/Tris (pH 7.4). Sections were then washed 5 times in BSA/Tris buffer (pH 7.4) and once in BSA/Tris buffer (pH 8.2) before being incubated for 1 hour at room temperature in either 10 or 15 nm gold-labelled goat anti-rabbit antibody (Biocell, Cardiff, U.K.) diluted 1:20 with BSA/Tris buffer (pH 8.2). The sections were finally washed thoroughly in DDW to remove any unbound gold antibody.

The sections were stained for 5 minutes with 2% aqueous uranyl acetate (BDH, Poole, U.K.) and washed thoroughly and dried before staining for 5 minutes with lead citrate (Appendix 7). They were finally washed thoroughly and dried before they were examined in a Philips EM 400T electron microscope.

Examination of the sections revealed that immunolabelled processes were covered by a high density of gold beads. A paired t-test was used to assess if the density of gold beads over immunolabelled processes was significantly different from background levels (Watson, 1988). A paired t-test was used instead of an unpaired t-test to avoid any effects of differences in labelling density between grids and different areas of the same section. The number of gold beads within a  $1\ \mu\text{m}^2$  area of an immunolabelled process was compared to the number of gold beads within a  $1\ \mu\text{m}^2$  area of an adjacent process that was covered by the background level of gold beads.

## **2.6. Confocal microscopical immunocytochemistry**

Transverse slices through the mesothoracic ganglia were processed for confocal microscopical immunocytochemistry using a modification of the method described by Sun et al., (1993). The free flotation method varied slightly depending on the antibody used, as they were conjugated to different fixatives. Therefore the specific methods used for each antibody have been described in detail in the appropriate chapters.

The nerve cord was exposed in the thoracic cavity (2.2.1) and fixed *in situ* for 5 minutes in the relevant fixative. The thoracic nerve cord was then removed and transferred to fresh fixative. The mesothoracic ganglion was sliced into serial transverse



sections (75-100  $\mu\text{m}$  thick) with a Vibroslice 725M tissue slicer (Campden Instruments Ltd, U.K.) (2.4.2) and then the slices were transferred to fresh fixative and fixed overnight at 4° C. The slices were rinsed thoroughly in 0.1 M Sorenson's phosphate buffer (pH 7.4) followed by BSA/Tris buffer (pH 7.4) (Appendix 7) containing 0.1% Triton X instead of Tween 20 (BSA/Tris/X). They were then incubated in 5% normal goat serum in BSA/Tris/X for 1 hour at room temperature to block any nonspecific staining before being incubated overnight at 4°C in their respective primary antibodies diluted with BSA/Tris/X (pH 7.4). Following incubation in the primary antibody the slices were washed first in BSA/Tris/X (pH 7.4) and then in BSA/Tris/X (pH 8.2). The tissue slices were then incubated overnight at 4°C with Rhodamine Red-labelled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, U.S.A.) diluted 1:200 with BSA/Tris/X (pH 8.2). From this stage onwards the slices were kept in the dark as the fluorescence of Rhodamine Red, like other fluorophores, diminishes in the presence of light. Following incubation in the secondary antibody the slices were washed thoroughly with BSA/Tris/X (pH 8.2) before dehydrating in an ethanol series (50%-100%) and clearing in methyl salicylate.

The slices were mounted on glass slides in Citifluor (Agar Aids, Stansted, U.K.) and examined under an inverted Axiophot microscope (Nikon U.K. Ltd, Surrey, U.K.) equipped with a Biorad Microradiance confocal system (Hemel Hempstead, U.K.) (excitation wavelength: 543 nm; detection with a longpass 600 nm filter). For each slice, optical sections were recorded at intervals of 0.5-1  $\mu\text{m}$  and projected to one plane.

All chemicals reported in this study were purchased from Sigma (Poole, U.K.) unless otherwise stated.

### **Chapter 3. Modulation of cholinergic synaptic transmission between the fSR and BA1 by GABAergic interneurons activated via muscarinic receptors.**

#### **3.1. INTRODUCTION.**

Since the early work of Dale (1914) the neurotransmitter acetylcholine has been studied extensively. The study of model cholinergic systems, such as the vertebrate neuromuscular junction and the electric organ of the electric ray *Torpedo californica*, have provided us with a vast amount of knowledge about the structure and function of cholinergic synapses and the receptors which mediate the responses to ACh. In addition to these more traditional cholinergic systems, the cholinergic systems of insects have also been used as models (Pitman, 1985; Sattelle, 1985; Sattelle and Breer, 1990; Baylis et al., 1996).

The insect CNS is an extremely rich source of ACh. It possesses a greater concentration of ACh and choline acetyltransferase (ChAT) (the enzyme which catalyses the conversion of choline to ACh) than the vertebrate CNS (Breer, 1981a; Sattelle and Breer, 1990). This alone does not provide conclusive evidence that ACh is a major neurotransmitter in insects but there is a vast amount of evidence from electrophysiological, neuropharmacological, genetic and molecular studies (see Pitman, 1985; Sattelle, 1985; Sattelle and Breer, 1990; Baylis et al., 1996), which indicates ACh does play a major role in insects. In addition, the fact that a number of insecticides which target the cholinergic system of insects are successful (Corbett et al., 1984) also indicates just how important ACh is in insects.

The cellular localisation of ACh in the insect CNS is unlike that in the vertebrate CNS. Insect motoneurons do not appear to be cholinergic as they lack ChAT activity (Emson et al., 1974), which is in contrast to vertebrate motoneurons. ChAT immunoreactivity is present however, in insect sensory neurons (Lutz and Tyrer, 1988). As sensory neurons terminate in the CNS, the synapses between insect sensory afferents and central neurons are attractive models to study central cholinergic synapses. The first cholinergic synapses between insect sensory afferents and identified central neurons to be studied in detail were between cercal afferents and giant interneurons in the cockroach *Periplaneta americana* (Sattelle et al., 1983; Blagburn and Sattelle, 1987b,c; Hue et al., 1989). Since then numerous other insect cholinergic synapses (tobacco hornworm hair afferent/proleg retractor motoneuron synapse, Trimmer and Weeks, 1989, 1993; locust fSR/BA1 synapse, Leitch et al., 1993, Leitch and Pitman, 1995; locust campaniform sensilla afferent/fast extensor tibiae motoneuron (FETi) synapse, Parker and Newland, 1995) have been used as models.

The study of insect cholinergic synapses has yielded important information that has contributed to our present knowledge of cholinergic synapses. The relatively large size and the morphology of some insect afferents has allowed microelectrode recordings to be made close to the presynaptic terminals of the sensory afferents (Blagburn and Sattelle, 1987a,c), which provides information on the presynaptic sequences that lead to ACh release. In addition, cholinergic synaptosomal fractions isolated from insect ganglia, which still possess the functional components of a presynaptic cholinergic terminal without the postsynaptic membranes, have been used to study presynaptic functions (Breer and Knipper, 1984; Knipper and Breer, 1989).

Genetic analysis of mutations associated with synapses in the fruit fly *Drosophila melanogaster* has also provided new information on the synaptic components vital for transmission. For example, analysis of mutants in the synaptotagmin gene, suggests that synaptotagmin acts as a calcium sensor and is involved in synaptic vesicle fusion (Littleton et al., 1994; Kelly, 1995; Littleton and Bellen, 1995). Not only are insect cholinergic synapses used to study presynaptic functions and ACh release, but the receptors which respond to ACh have also been examined. Advances in molecular techniques have allowed cholinergic receptors and receptor subunits to be cloned from insects and have provided us with information on the pharmacology, structure and function of cholinergic receptors (Onai et al., 1989; Shapiro et al., 1989; Marshall et al., 1990; Blake et al., 1993).

In the vertebrate CNS, cholinergic receptors can be divided into two main classes. The nicotinic cholinergic receptors are stimulated by nicotine and the muscarinic cholinergic receptors are stimulated by muscarine. Nicotinic cholinergic receptors are macromolecules comprising five transmembrane subunits forming a channel. When ACh binds to the  $\alpha$  subunits, the receptor undergoes a conformational change which opens the channel, allowing ions to pass through (Changeux, 1981; Raftery et al., 1980). As the nicotinic receptor is directly associated with the ion channel the response which it mediates is fast. Muscarinic receptors, on the other hand, are not directly associated with ion channels so therefore induce a slower response.

Muscarinic receptors mediate their responses via G-proteins, which act on effector enzymes e.g. adenylate cyclase or ion channels to increase or decrease intracellular second messengers e.g. cAMP (see Felder, 1995). The second messengers either act on the ion channel itself or activate a protein kinase, which phosphorylates either the

ion channel protein or a regulatory protein that acts on the ion channel. Like all G-protein-coupled receptors, muscarinic cholinergic receptors are a single polypeptide chain that spans the membrane seven times (Kubo et al., 1986). Molecular cloning has revealed 5 subtypes of the vertebrate muscarinic cholinergic receptor. Although the amino-acid sequence in all 5 subtypes is highly conserved with them sharing at least 90% amino-acid identity (Bonner, 1989), there are marked differences in the hydrophilic cytoplasmic domains, which are responsible for G-protein coupling. It is not surprising therefore, that the different muscarinic subtypes may be coupled to different second messengers (see Felder, 1995).

Nicotinic and muscarinic cholinergic receptors can be distinguished pharmacologically using general agonists which activate cholinergic receptors, and general antagonists which block the cholinergic receptors. For example, the general antagonist  $\alpha$ -bungarotoxin is more effective at blocking nicotinic cholinergic receptors than the general antagonist atropine, whereas atropine is more effective at blocking muscarinic cholinergic receptors than  $\alpha$ -bungarotoxin. Different subtypes of cholinergic receptors can also be distinguished using selective agonists and antagonists. For example, the selective antagonist pirenzepine has a high affinity for  $M_1$  muscarinic receptors (Hammer et al., 1980) whereas, the antagonist methoctramine is selective for  $M_2$  muscarinic receptors.

The agonists and antagonists used in vertebrates to distinguish between different types of cholinergic receptors have also been used in binding and electrophysiological studies to characterise cholinergic receptors in insects (Table 3.1). Unlike the vertebrate CNS, there appear to be three main classes of cholinergic receptors in the

**Table 3.1. Pharmacological agents used to characterise insect cholinergic receptors.**

Receptor Type	Agonists	Refs.	Antagonists	Refs.
Nicotinic	nicotine	1, 2, 3, 4, 5, 6, 7	$\alpha$ -bungarotoxin <i>d</i> -tubocarinine mecamylamine decamethonium gallamine	1, 2, 3, 4, 5, 8, 9, 10 1, 2, 4, 6, 10 6, 7, 10 2, 4, 7, 11 1, 2, 3, 4, 9
Muscarinic	muscarine	7, 10, 11, 19	atropine	4, 7, 9, 10, 11, 12, 14, 15, 18, 19
	oxotremorine	7, 10, 11	scopolamine	4, 9, 10, 12, 14, 19
	arecoline	17, 19	QNB	4, 9, 11, 12, 13, 14, 15, 16, 19
	pilocarpine	14, 18		
M <sub>1</sub>	McN-A-343	11	pirenzipine	11, 15, 16, 20, 21, 22
M <sub>2</sub>			methoctramine AF-DX 116	9, 16, 17 17
M <sub>3</sub>			4-DAMP	15, 22

References: (1) Dudai, 1978; (2) Breer, 1981b; (3) Jones et al., 1981; (4) Lummis and Sattelle, 1985; (5) Bicker and Kreissl, 1994; (6) Blagburn and Sattelle, 1987b; (7) Parker and Newland, 1995; (8) Sattelle et al., 1983; (9) Leitch and Pitman, 1995; (10) Trimmer and Weeks, 1989; (11) David and Pitman, 1993; (12) Dudai and Ben-Barak, 1977; (13) Meyer and Edwards, 1980; (14) Jones and Sumikawa, 1981; (15) Abdallah et al., 1991; (16) Qazi et al., 1996; (17) Le Corrone et al., 1991; (18) Hue et al., 1989; (19) Le Corrone and Hue, 1993; (20) Knipper and Breer, 1988; (21) Trimmer and Weeks, 1993; (22) Blake et al., 1993.

Abbreviations: QNB, quinuclidinyl benzilate; McN-A-343, (4-hydroxy-2-butyryl) trimethyl-ammonium chloride; AF-DX 116, 11-((2-((dimethylamino)-methyl)1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepin-6 one; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide.

insect CNS. One class of insect cholinergic receptor has a pharmacological profile like the vertebrate nicotinic cholinergic receptor (Breer, 1981b; Jones et al., 1981; Lummis and Sattelle, 1985; Sattelle et al., 1983; Blagburn and Sattelle, 1987b). Nicotine is the most effective agonist at this class of receptor and  $\alpha$ -bungarotoxin the most effective antagonist (Kerkut et al., 1969; David and Sattelle, 1984; Benson, 1992). Binding studies using [ $^3$ H]  $\alpha$ -bungarotoxin reveal that  $\alpha$ -bungarotoxin binding sites are present in the CNS of the fruit fly (Dudai, 1978), locust (Breer, 1981b), house fly (Jones et al., 1981) cockroach (Lummis and Sattelle, 1985) and cricket (Meyer and Reddy, 1985). Purification of the  $\alpha$ -bungarotoxin binding protein and reconstitution in a planar lipid bilayer, shows that the  $\alpha$ -bungarotoxin binding protein is a functional acetylcholine receptor channel i.e. a nicotinic cholinergic receptor (Hanke and Breer, 1986).

A second class of insect cholinergic receptor identified in the insect CNS possesses a pharmacological profile which is similar to the vertebrate muscarinic cholinergic receptor profile e.g. it has a high affinity for the muscarinic antagonist quinuclidinyl benzilate (QNB) (Dudai and Ben-Barak, 1977; Meyer and Edwards, 1980; Breer, 1981a; Jones and Sumikawa, 1981; Lummis and Sattelle, 1985; Abdallah et al., 1991; Qazi et al., 1996). Binding studies using [ $^3$ H] QNB show that muscarinic cholinergic receptors are present in the CNS of the fruit fly (Dudai and Ben-Barak, 1977), locust (Breer, 1981a), house fly (Jones and Sumikawa, 1981; Abdallah et al., 1991), cockroach (Lummis and Sattelle, 1985; Abdallah et al., 1991), cricket (Meyer and Edwards, 1980), honey bee (Abdallah et al., 1991) and the moth (Qazi et al., 1996). Comparison of the number of [ $^3$ H]  $\alpha$ -bungarotoxin binding sites with the number of [ $^3$ H] QNB binding sites, indicates that the insect CNS contains a greater

number of nicotinic than muscarinic cholinergic receptors (Breer, 1981a; Lummis and Sattelle, 1985).

The third class of cholinergic receptor identified in the insect CNS possesses both muscarinic and nicotinic receptor characteristics (Lapied et al., 1990; David and Pitman, 1993, 1995; Grolleau et al., 1996). Cholinergic receptors with a 'mixed' pharmacology have been identified on the fast coxal depressor ( $D_f$ ) motoneuron (David and Pitman, 1993, 1995, 1996a,b,c) and on the dorsal unpaired median (DUM) neurons of the cockroach (Lapied et al., 1990; Grolleau et al., 1996). The 'mixed' pharmacology receptors on  $D_f$  respond to both nicotinic and muscarinic agonists but are insensitive to the nicotinic antagonist  $\alpha$ -bungarotoxin (David and Pitman, 1993). The 'mixed' pharmacology receptors on the DUM neurons also respond to nicotine but are blocked by both nicotinic and muscarinic antagonists (Lapied et al., 1990; Grolleau et al., 1996).

As in the vertebrate CNS, the different types of cholinergic receptors in the insect CNS appear to have different physiological functions. Nicotinic cholinergic receptors are generally present on the postsynaptic membrane of a cholinergic synapse (Sattelle et al., 1983; Parker and Newland, 1995; Leitch and Pitman, 1995). Postsynaptic potentials, evoked in response to ACh released from presynaptic afferents, are inhibited by nicotinic antagonists (Sattelle et al., 1983; Leitch and Pitman, 1995). The depolarisations mediated by these nicotinic cholinergic receptors on postsynaptic neurons appear to be due to the postsynaptic neuron becoming more permeable to  $\text{Na}^+$  ions (Kerkut et al., 1969). Nicotinic receptors have also been identified on the presynaptic neurons of a cholinergic synapse (Blagburn and Sattelle, 1987b). These



receptors are thought to be located on the axon of the presynaptic neuron within the neuropil of the ganglion, but their functional role is has not been determined.

The 'mixed' pharmacology receptors identified on the cockroach  $D_f$  motoneuron (David and Pitman, 1993, 1995, 1996a,b,c) and on the cockroach DUM neurons (Lapied et al., 1990; Grolleau et al., 1996) coexist with nicotinic (David and Sattelle, 1984), muscarinic (Lapied et al., 1992; Bai and Sattelle, 1994) and GABA receptors (Sattelle et al., 1988). It appears that the 'mixed' pharmacology receptors modulate the currents evoked by the other receptors in the same neuron. Activation of the 'mixed' pharmacology receptors on  $D_f$  results in a biphasic response which consists of an initial outward current, that appears to be due to an increase in an outward  $Ca^{2+}$ -activated  $K^+$  current ( $I_{K,Ca}$ ), and a delayed inward current, which appears to be due to the reduction of a  $Ca^{2+}$  current which subsequently causes a decrease in  $I_{K,Ca}$  (David and Pitman, 1995, 1996b). The response to the activation of the 'mixed' pharmacology receptors on  $D_f$  and the cockroach DUM neurons is not due to a change in cAMP (David and Pitman, 1996a) but appears to be due to a rise in intracellular  $Ca^{2+}$  (David and Pitman, 1996b; Grolleau et al., 1996). The rise in intracellular  $Ca^{2+}$  following the activation of the 'mixed' pharmacology receptors modulates nicotinic acetylcholine and GABA currents in  $D_f$ , indicating that the intracellular messengers of the 'mixed' pharmacology receptors may modulate nicotinic and GABA gated ion channels (David and Pitman, 1996c).

Muscarinic cholinergic receptors appear to have a number of functional roles in the insect CNS (see Trimmer, 1995). Some muscarinic receptors are involved in evoking motor patterns in the insect CNS (Ryckebusch and Laurent, 1993; Büschges et al.,

1995; Johnston and Levine, 1996; Rast and Bräunig, 1997). Long-lasting rhythmic activity can be evoked in the leg motoneurons of the locust (Ryckebusch and Laurent, 1993) and the stick insect (Büschges et al., 1995) by applying the muscarinic agonist pilocarpine. The coupling of the rhythmic activity between the motoneuron pools to antagonistic leg muscles, evoked by pilocarpine, resembles the motor output during walking (Ryckebusch and Laurent, 1993; Büschges et al., 1995). Other motor patterns evoked by pilocarpine include a rhythmic pattern of activity in the motoneurons which innervate the body wall muscles of *Manduca sexta* larvae, which is similar to the motor pattern associated with crawling (Johnston and Levine, 1996) and a rhythmic pattern of activity in the locust mandibular motoneurons, which resembles the motor output during feeding (Rast and Bräunig, 1997). The motor patterns evoked by pilocarpine can be blocked by the muscarinic antagonist atropine, indicating that the motor pattern is mediated via muscarinic receptors (Ryckebusch and Laurent, 1993; Büschges et al., 1995; Johnston and Levine, 1996; Rast and Bräunig, 1997).

The induction of motor patterns, by the muscarinic agonist pilocarpine, may be explained by examining the functional roles of muscarinic receptors on individual neurons. Activation of postsynaptic muscarinic receptors located on unidentified neurons in the locust (Benson, 1992), the proleg retractor motoneurons in *Manduca sexta* (Trimmer and Weeks, 1993) and the ventral giant interneuron in the cockroach (Le Corrionc and Hue, 1993) evokes a slow depolarisation. The slow depolarisation reduces the spike threshold and therefore increases the excitability of the neurons. An increase in the excitability increases the probability that the ventral giant interneuron and proleg retractor motoneuron cercal afferent stimulation will evoke spikes in

response to sensory afferent stimulation (Le Corrionc and Hue, 1993; Trimmer and Weeks, 1993). This mechanism of gain control could therefore counteract sensory depression resulting from repeated stimulation of the sensory afferents.

The ionic basis of the slow depolarisations evoked by the activation of these postsynaptic muscarinic receptors appears to be due a reduction in a  $K^+$  conductance (Benson, 1992; Le Corrionc and Hue, 1993). The reduction in the  $K^+$  conductance appears to be independent of  $Ca^{2+}$  in unidentified locust neurons (Benson, 1992) and  $Ca^{2+}$  dependent in the cockroach ventral giant interneuron (Le Corrionc and Hue, 1993).

The pharmacology of the muscarinic receptors on postsynaptic neurons resembles the pharmacological profiles of vertebrate  $M_1$  and  $M_3$  muscarinic receptors (Benson, 1992; Le Corrionc and Hue, 1993; Trimmer and Weeks, 1993). This is intriguing as  $M_1$  and  $M_3$  muscarinic receptors in vertebrates are also mostly localised postsynaptically (Buckley et al., 1988). The  $M_2$  muscarinic receptor subtype on the other hand, is predominantly presynaptic in the vertebrate CNS (Buckley et al., 1988; Bellingham and Berger, 1996).

Presynaptically located muscarinic receptors have also been identified in the insect CNS (Breer and Knipper, 1984; Hue et al., 1989; Knipper and Breer, 1989; Trimmer and Weeks, 1989; Le Corrionc and Hue, 1991; Leitch and Pitman, 1995; Parker and Newland, 1995), and their pharmacological profiles most closely resemble vertebrate  $M_2$  muscarinic receptors (Knipper and Breer, 1988; Le Corrionc et al., 1991; Leitch and Pitman, 1995). Presynaptic muscarinic receptors were first identified in a locust

(*Locusta migratoria*) synaptosomal preparation (Breer and Knipper, 1984; Knipper and Breer, 1989). Activation of the muscarinic autoreceptors with muscarinic agonists down-regulated the release of ACh from the synaptosomes and application of muscarinic antagonists potentiated the release of ACh. As these muscarinic autoreceptors displayed a low affinity for the M<sub>1</sub> muscarinic receptor antagonist pirenzepine, it was suggested that ACh release from the synaptosomes was mediated by M<sub>2</sub> muscarinic receptors (Knipper and Breer, 1988).

Subsequent electrophysiological studies on a number of insect cholinergic synapses (cockroach cercal afferent/giant interneuron synapses, Hue et al., 1989, Le Corrionc et al., 1991; tobacco hornworm hair afferent/proleg retractor motoneuron synapses, Trimmer and Weeks, 1989; locust fSR/BA1 synapses, Leitch and Pitman, 1995; locust campaniform sensilla afferent/FETi synapses, Parker and Newland, 1995) have provided direct evidence that presynaptic muscarinic receptors play a role in modulating ACh release at cholinergic synapses. The amplitude of EPSPs, evoked by the stimulation of a cholinergic afferent and recorded from the postsynaptic neuron, increases following the application of general muscarinic antagonists (Hue et al., 1989; Trimmer and Weeks, 1989; Leitch and Pitman, 1995; Parker and Newland, 1995). This indicates that muscarinic receptors normally involved in inhibiting synaptic transmission between the sensory afferent and postsynaptic neuron are being blocked. The increase in the amplitude of the EPSPs does not appear to be due to the muscarinic antagonist affecting the postsynaptic neurons, indicating its effects are mediated via presynaptic muscarinic receptors (Hue et al., 1989; Trimmer and Weeks, 1989; Leitch and Pitman, 1995; Parker and Newland, 1995). Agonists and antagonists selective for different muscarinic receptor subtypes were used to examine

the pharmacological profile of the presynaptic muscarinic autoreceptors (Le Corrone et al., 1991; Leitch and Pitman, 1995). As the muscarinic antagonists selective for  $M_2$  muscarinic receptors (e.g. methoctramine and AF-DX 116) were most effective at blocking the muscarinic autoreceptors and the muscarinic antagonists selective for  $M_1$  and  $M_3$  muscarinic receptors (e.g. pirenzepine and 4-DAMP) were ineffective, it was suggested that  $M_2$  muscarinic receptors down-regulated ACh release from the presynaptic terminals of the insect sensory afferents (Le Corrone et al., 1991; Leitch and Pitman, 1995).

The precise intracellular mechanisms evoked when insect presynaptic muscarinic receptors are stimulated are unknown, although Knipper and Breer (1988) did report that the activation of the muscarinic autoreceptors on locust synaptosomes significantly reduced the accumulation of cAMP (Knipper and Breer, 1988). This is consistent with their suggestion that the muscarinic autoreceptors in the synaptosomal preparation are  $M_2$  muscarinic receptors as vertebrate  $M_2$  muscarinic receptors are negatively coupled to the enzyme adenylate cyclase (Watson et al., 1984; Hosey, 1992).

It has generally been assumed that the presynaptic muscarinic receptors involved in the down-regulation of ACh release from a cholinergic neuron are located on the presynaptic terminals of the cholinergic neuron and act as autoreceptors. It is possible however, that the muscarinic receptors may be located on inhibitory interneurons which receive inputs from collaterals of the cholinergic neuron. In this scenario, the ACh released after stimulation of the cholinergic neuron would activate the

muscarinic receptors on the interneurons, which would in turn inhibit the presynaptic terminals of the cholinergic neuron and thereby reduce further release of ACh.

The main inhibitory neurotransmitter implicated in presynaptic inhibition in invertebrates is GABA (see Watson, 1992a). Evidence that GABA is involved in presynaptic inhibition has been provided by ICC (Watson, 1990; Watson and England, 1991; Watson et al., 1991, 1993; Watson and Pflüger, 1994; Pflüger and Watson, 1995) and electrophysiological studies (Hue and Callec, 1983; Burrows and Laurent, 1993). ICC has revealed that a number of insect neurons receive synaptic inputs from neurons immunoreactive for GABA (locust cercal afferents, Watson, 1990; campaniform sensilla, Watson and England, 1991; hair plates, Watson et al., 1991; femoral chordotonal organ afferents, Watson et al., 1993; prosternal filiform afferents, Watson and Pflüger, 1994; dorsal unpaired median neurons, Pflüger and Watson, 1995). Frequently the GABA-immunoreactive neurons make synaptic inputs onto the terminals of the neurons, which are filled with synaptic vesicles (Watson et al., 1991, 1993; Watson and Pflüger, 1994), indicating that neurotransmitter release from the terminals is closely controlled by the GABA-immunoreactive neurons.

Direct evidence that GABA is involved in presynaptically inhibiting insect sensory afferents has been demonstrated in electrophysiological studies (Hue and Callec, 1983; Burrows and Laurent, 1993). At the cockroach cercal afferent/ giant interneuron synapses, repetitive stimulation of the cockroach cercal afferents results in a decrease in the amplitude of EPSPs recorded from the giant interneuron (Hue and Callec, 1983). The decrease in the EPSP amplitude is thought to be due to a chloride-dependent hyperpolarisation recorded from the cercal afferent, which

inhibits ACh release. As the chloride-dependent hyperpolarisation can be mimicked by GABA, it indicates that GABA presynaptically inhibits ACh release from the cercal afferents (Hue and Callec, 1983). Locust femoral chordotonal organ (FeCO) afferents are also presynaptically inhibited by interposing GABAergic interneurons, which reduces the ability of the afferent to spike (Burrows and Laurent, 1993; Burrows and Matheson, 1994). The presynaptic inhibition of FeCO afferents, however, is due to depolarisation. These results indicate that the presynaptic inhibition of both the locust FeCO afferents and cockroach cercal afferents is mediated via GABA receptors.

The GABA receptors involved in presynaptically inhibiting the insect sensory afferents have not been characterised but a recent study examining the presynaptic inhibition of a crayfish opener neuromuscular junction revealed that both GABA<sub>A</sub> and GABA<sub>B</sub> receptors were involved, and each evoked a different mechanism of presynaptic inhibition (Fischer and Parnas, 1996). In the insect CNS two main types of GABA receptors have been identified; picrotoxin-sensitive GABA receptors (Benson, 1988; Buckingham et al., 1994; Aydar et al., 1995) and picrotoxin-insensitive GABA receptors (Hue, 1991; Bai and Sattelle, 1995). Picrotoxin-sensitive GABA receptors are chloride-dependent ionotropic receptors and most have pharmacological profiles similar to GABA<sub>A</sub> receptors but are insensitive to bicuculline (Benson, 1988; Buckingham et al., 1994). Some picrotoxin-sensitive GABA receptors however, have pharmacological profiles that are not like those of GABA<sub>A</sub> (Aydar et al., 1995). Picrotoxin-insensitive GABA receptors have pharmacological profiles similar to GABA<sub>B</sub> receptors but they are insensitive to baclofen (Hue, 1991; Bai and Sattelle, 1995).

The GABA-induced presynaptic inhibition of insect afferents (Hue and Callec, 1983; Burrows and Laurent, 1993) appears to be chloride dependent. The GABA-induced presynaptic inhibition of the locust FeCO afferents is antagonised by picrotoxin, which blocks GABA receptor chloride channels (Burrows and Laurent, 1993) and lowering the chloride levels in the saline reverses the GABA-induced hyperpolarisation that presynaptically inhibits cockroach cercal afferents (Hue and Callec, 1983). The fact that the presynaptic inhibition of the insect sensory afferents is chloride-dependent, indicates that GABA<sub>A</sub> receptors are involved.

There is evidence that some insect cholinergic synapses (cockroach cercal afferent/giant interneuron synapses, Hue and Callec, 1983, Hue et al., 1989, Le Corrionc et al., 1991; locust campaniform sensilla afferents/ FETi motoneuron synapses, Watson and England, 1991, Parker and Newland, 1995) are presynaptically modulated by both muscarinic receptors and GABAergic interneurons. It is possible therefore, that at least some of the muscarinic receptors may be located on GABAergic interneurons. Evidence to support this comes from a study (Le Corrionc et al., 1991) on the cockroach cercal afferent/giant interneuron preparation which suggests that the GABAergic interneurons involved in the modulation of ACh release from the cercal afferent terminals are partly activated by muscarinic agonists.

To investigate the possibility that presynaptic muscarinic receptors involved in modulating ACh release from sensory afferent terminals may be located on GABAergic interneurons, the locust fSR/BA1 preparation was used in this study. This synapse which is part of a sensory pathway involved in locust flight is cholinergic and is



modulated by muscarinic receptors located presynaptically (Leitch and Pitman, 1995). It differs from the other insect sensory afferent/central neurons synapses, which are modulated by presynaptic muscarinic receptors, as the fSR is a single sensory neuron rather than part of a sensory array. One fSR is associated with the hinge of each forewing (Gettrup, 1962) and makes monosynaptic connections with depressor motoneurons, including BA1 in the CNS (Burrows, 1975). The aims of the experiments conducted in this chapter were; a) to confirm that muscarinic receptors are involved in modulating synaptic transmission at the fSR/BA1 synapse in the locust *Locusta migratoria*, b) to establish if the muscarinic receptors are located presynaptically, c) to examine the possibility that GABAergic interneurons are also involved in modulating the fSR/BA1 synapse and d) to determine if at least some of the muscarinic receptors are located on GABAergic interneurons. The majority of this work has been published in abstracts (Judge and Leitch, 1998; Leitch et al., 1998) and in a paper (Judge and Leitch, 1999c).

### **3.2. MATERIALS AND METHODS**

Electrophysiology was used to determine if GABAergic interneurons, activated via muscarinic receptors, presynaptically modulate synaptic transmission between the fSR and BA1 in the locust. Electrically-evoked EPSPs and cholinergic responses were elicited and recorded from BA1 in an isolated locust thoracic nerve cord preparation, using the methods described in detail in Chapter 2.2.

The effects of the following compounds were tested: atropine sulfate, picrotoxin,

(-)-scopolamine (scopine tropate) hydrochloride. All reagents were purchased from Sigma (Poole, U.K.). The compounds were applied at concentrations that were shown to be effective in other insect preparations.

### **3.3. RESULTS.**

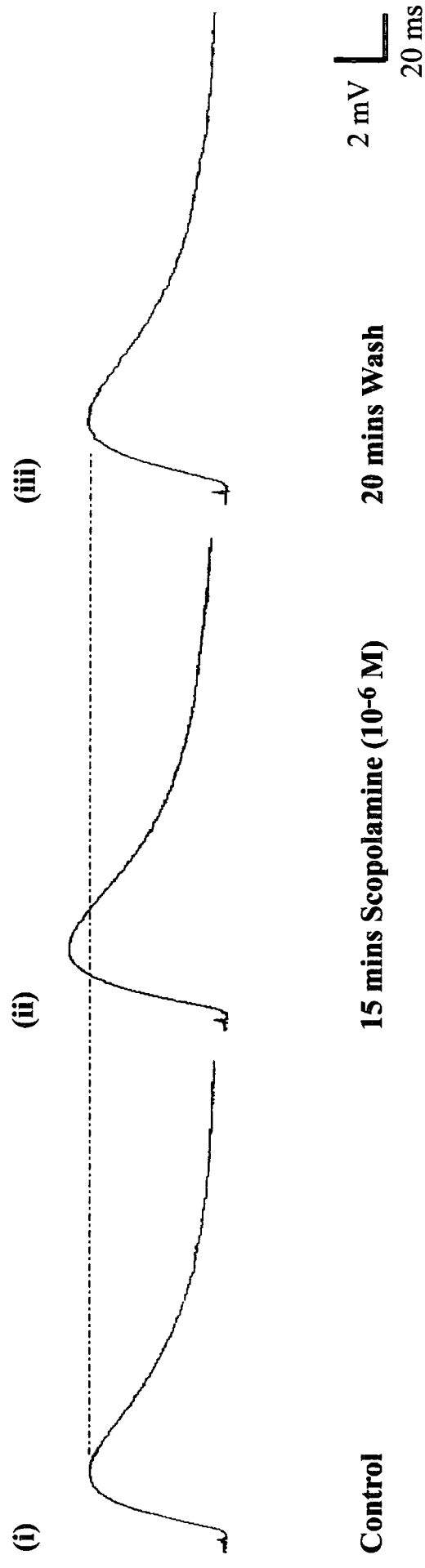
#### **3.3.1. Effects of muscarinic cholinergic antagonists on electrically-evoked EPSPs recorded from BA1**

ACh release from the fSR terminals in the locust *Schistocerca gregaria* is down-regulated by muscarinic cholinergic receptors located presynaptically (Leitch and Pitman, 1995). To confirm that presynaptic muscarinic cholinergic receptors are involved in the modulation of ACh release from the fSR terminals in the locust *Locusta migratoria*, the effects of general muscarinic cholinergic antagonists on synaptic transmission between the fSR and the motoneuron BA1, were investigated (Figures 3.1-3.4).

Synaptic transmission between the fSR and BA1 was elicited by electrically stimulating the fSR axon, which evoked EPSPs in BA1. Under control conditions electrical stimulation of the fSR evoked EPSPs in BA1 in the locust *Locusta migratoria* with a mean amplitude of  $4.38 \pm 0.33$  mV (mean  $\pm$  SEM;  $n = 45$ ) and a duration of  $136.95 \pm 5.9$  ms (mean  $\pm$  SEM;  $n = 45$ ) (Figures 3.1i, 3.2i). Following the bath application of either the general muscarinic antagonist scopolamine ( $10^{-6}$  M) (Figure 3.1ii) or atropine ( $10^{-8}$ - $10^{-6}$  M) (Figure 3.2ii) the amplitude and the duration of the electrically-evoked EPSPs recorded from BA1 increased. The effects of the muscarinic antagonists on the

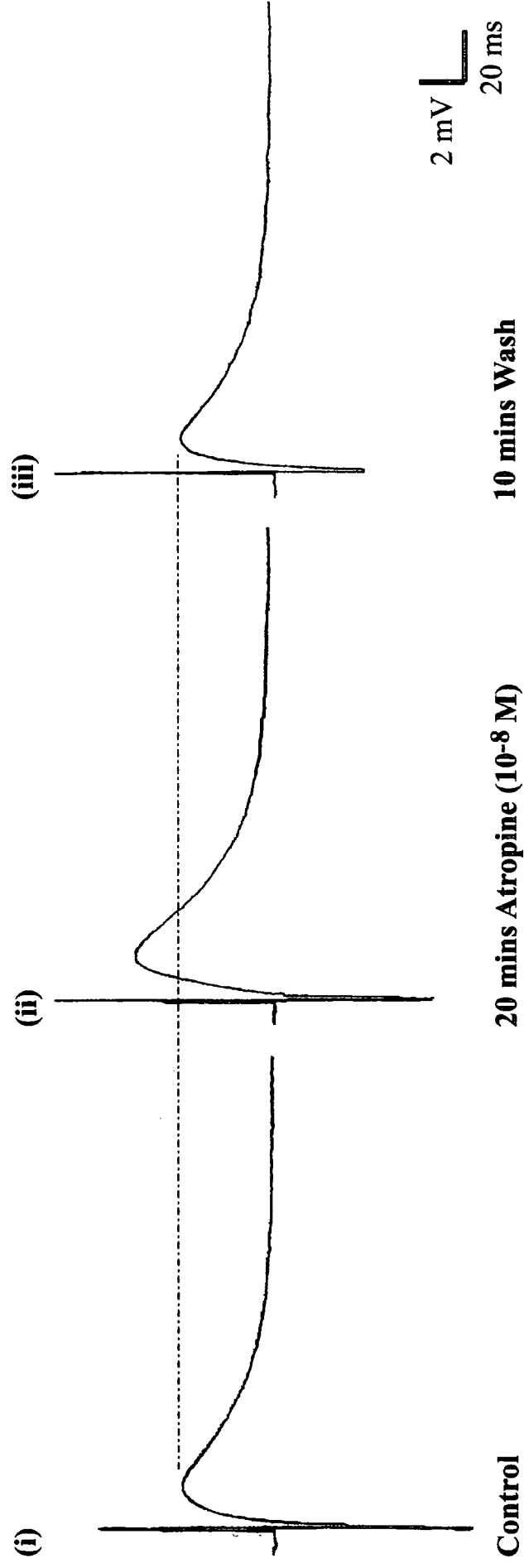
**Figure 3.1. Effects of Scopolamine ( $10^{-6}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the selective electrical stimulation of the fSR axon. (ii) The muscarinic antagonist scopolamine ( $10^{-6}$  M) caused an increase in the EPSP amplitude and duration which was reversible upon washing (iii). Each trace is an average of 8 successive electrically-evoked EPSPs.

**Figure 3.1. Effects of Scopolamine ( $10^{-6}$  M) on electrically-evoked EPSPs.**



**Figure 3.2. Effects of Atropine ( $10^{-8}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the selective electrical stimulation of the fSR axon. (ii) The muscarinic antagonist atropine ( $10^{-8}$  M) caused an increase in the EPSP amplitude and duration. (iii) This increase was reversible upon washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

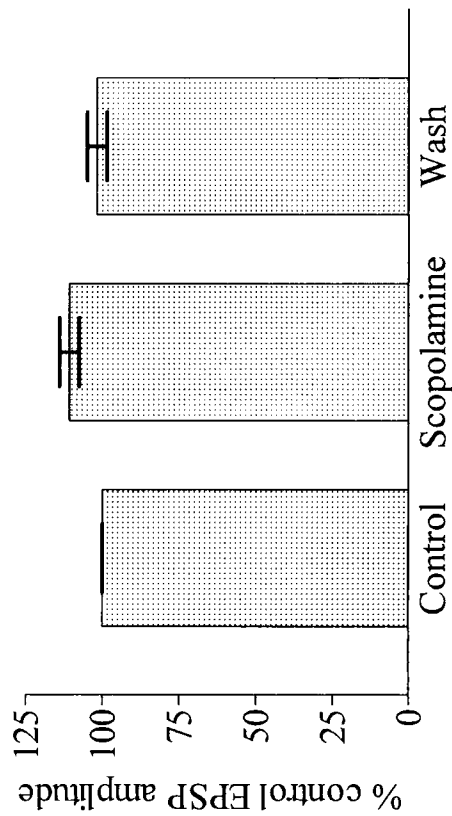
Figure 3.2. Effects of Atropine ( $10^{-8}$  M) on electrically-evoked EPSPs.



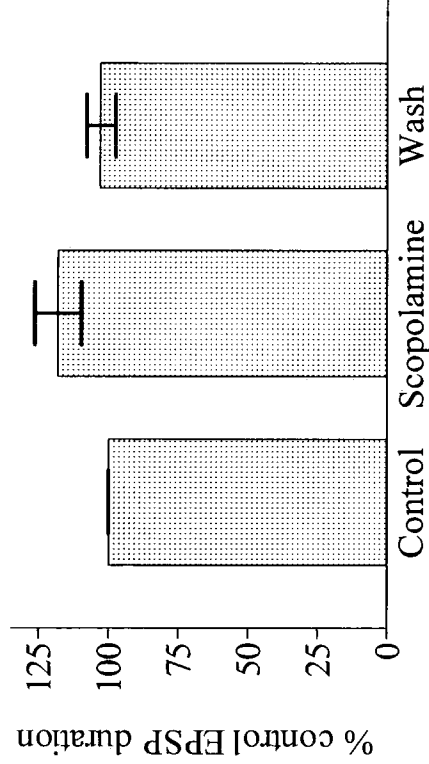
EPSP amplitude were reversed by washing the preparation with drug-free saline (Figure 3.1iii, 3.2iii).

Bath application of scopolamine ( $10^{-6}$  M) caused the EPSP amplitude recorded from BA1 to increase by a maximum mean percentage of 10.8 % (+ SEM 3.1; -SEM 3.3;  $p = 0.008$ ;  $n = 10$ ) (Figure 3.3A). The duration of the EPSPs recorded from BA1 increased by a maximum mean percentage of 18.2% (+ SEM 8.2; -SEM 8.5;  $p = 0.04$ ;  $n = 10$ ) following the bath application of scopolamine ( $10^{-6}$  M) (Figure 3.3B). Bath application of atropine ( $10^{-8}$  M) caused the EPSP amplitude recorded from BA1 to increase by a maximum mean percentage of 23 % (+ SEM 6.5; -SEM 7;  $p = 0.021$ ;  $n = 5$ ) (Figure 3.4A) and caused the EPSP duration to increase by a maximum mean percentage of 47.3 % (+ SEM 18.7; -SEM 21.5;  $n = 5$ ) (Figure 3.4B). In one preparation, it was necessary to increase the concentration of atropine to  $10^{-6}$  M to induce an increase in the EPSP amplitude of 22.2% and an increase in the EPSP duration of 33%. The increase in the EPSP amplitude and duration in the presence of the muscarinic antagonists reversed after washing the preparation with drug-free saline.

The effects of both scopolamine ( $10^{-6}$  M) and atropine ( $10^{-8}$ - $10^{-6}$  M) on the electrically-evoked EPSP amplitude began within 5 minutes of application. Within 25 minutes of scopolamine ( $10^{-6}$  M) application ( $n = 10$ ), 10 minutes of atropine ( $10^{-6}$  M) application ( $n = 1$ ) and 30 minutes of atropine ( $10^{-8}$  M) application ( $n = 5$ ), the increase in EPSP amplitude had generally reached a maximum. The effects of scopolamine on the EPSP amplitude and duration reversed within 10-20 minutes of washing the preparation with drug-free saline and the effects of atropine on the EPSP amplitude and duration reversed within 15 minutes of washing.

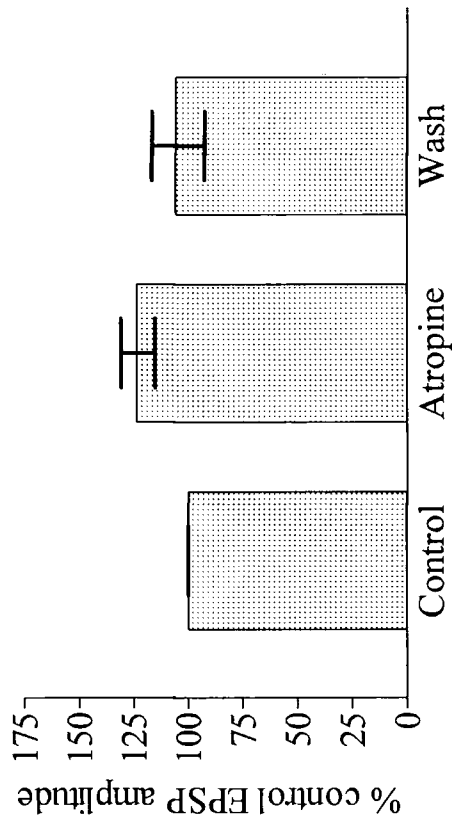


**Figure 3.3A. Maximum effect of Scopolamine ( $10^{-6}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of scopolamine and following wash ( $n = 10$ ). The amplitude is given as a percentage of the control amplitude.

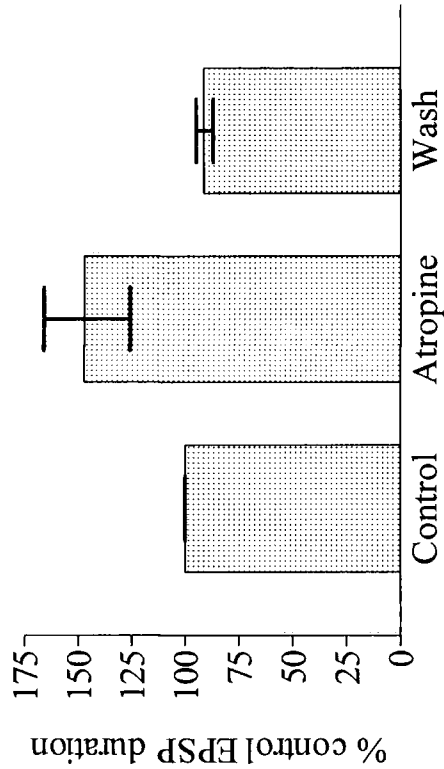


**Figure 3.3B. Maximum effect of Scopolamine ( $10^{-6}$  M) on EPSP duration.** Graphical representation of the pooled data from 10 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of scopolamine and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.





**Figure 3.4A. Maximum effect of Atropine ( $10^{-8}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of atropine and following wash ( $n = 5$ ). The amplitude is given as a percentage of the control amplitude.



**Figure 3.4B. Maximum effect of Atropine ( $10^{-8}$  M) on EPSP duration.** Graphical representation of the pooled data from 5 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of atropine and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.

Following the application of the muscarinic antagonists, the membrane potential and input resistance of BA1 only fluctuated slightly by  $0.7 \pm 0.6$  mV (mean  $\pm$  SEM) and  $1 \pm 1$  M $\Omega$  (mean  $\pm$  SEM), respectively. A two-tailed paired t-test showed that the small changes in the membrane potential ( $p = 0.31$ ) and input resistance ( $p = 0.5$ ) were statistically insignificant. This indicates that the effects of the muscarinic antagonists on the electrically-evoked EPSPs are not due to changes in the membrane conductance of BA1.

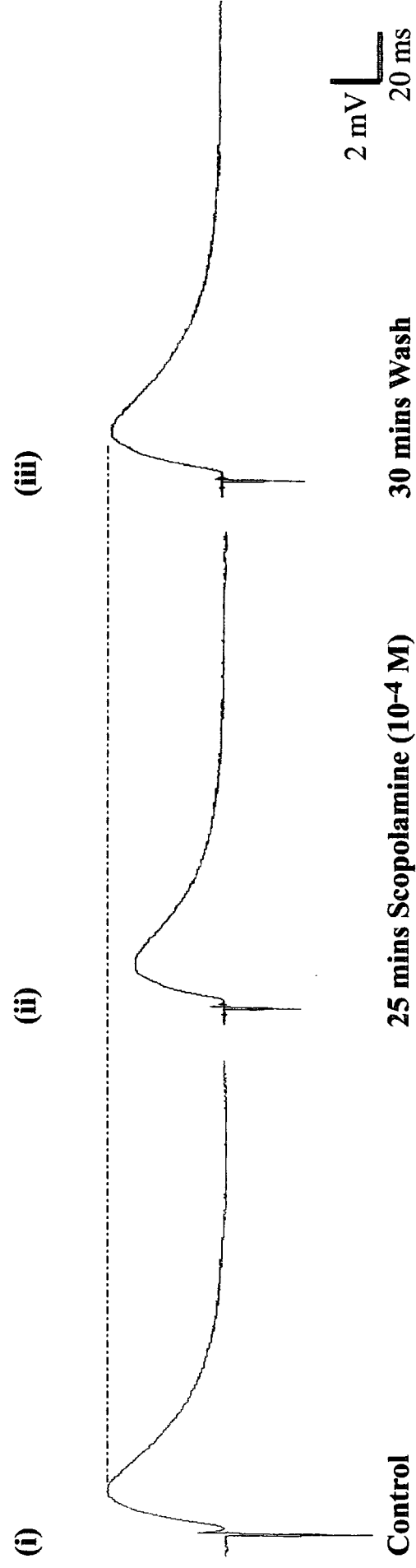
The above results show that bath application of scopolamine ( $10^{-6}$  M) and atropine ( $10^{-8}$  M- $10^{-6}$  M) causes a reversible increase in the amplitude and duration of electrically-evoked EPSPs recorded from BA1 in the locust *Locusta migratoria*. The increase in the EPSP amplitude and duration indicates that muscarinic receptors, that are normally involved in modulating synaptic transmission between the fSR and BA1, are being blocked by the muscarinic antagonists.

### **3.3.2. Effects of higher concentrations of muscarinic cholinergic antagonists on electrically-evoked EPSPs recorded from BA1**

The action of the muscarinic antagonists on electrically-evoked EPSPs, was found to be concentration dependent. At the concentration of  $10^{-4}$  M, the muscarinic antagonist scopolamine caused a decrease in the amplitude and duration of electrically-evoked EPSPs recorded from BA1 (Figure 3.5A). Bath application of scopolamine ( $10^{-4}$  M) caused the EPSP amplitude recorded from BA1 to decrease by a maximum mean percentage of 22.2 % (+ SEM 2.3; -SEM 2.4;  $p < 0.0001$ ;  $n = 14$ ) (Figure 3.5B) and the

**Figure 3.5A. Effects of Scopolamine ( $10^{-4}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) Higher concentrations of the muscarinic antagonist scopolamine ( $10^{-4}$  M) caused a decrease in the EPSP amplitude and duration. (iii) This decrease was reversible after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

**Figure 3.5A. Effects of Scopolamine ( $10^{-4}$  M) on electrically-evoked EPSPs.**



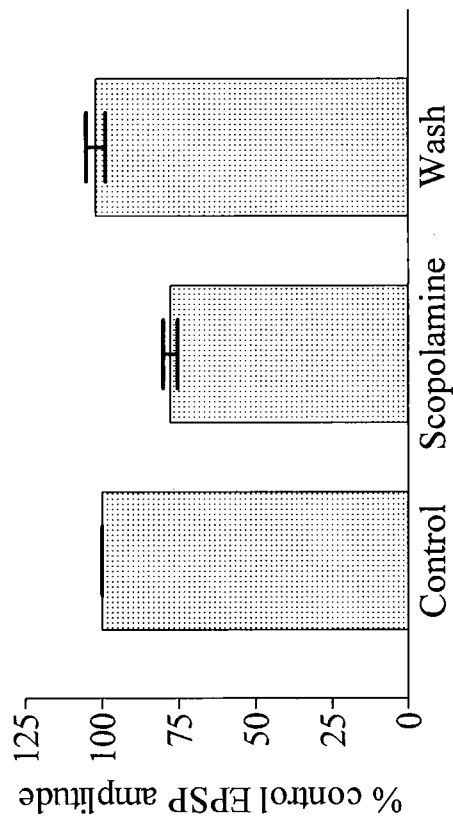
EPSP duration to decrease by a maximum mean percentage of 9.1 % (+ SEM 1.7; - SEM 1.8) (Figure 3.5C). The decrease in the EPSP amplitude and duration in the presence of scopolamine ( $10^{-4}$  M) reversed after washing the preparation with drug-free saline (Figure 3.5). The effects of the muscarinic antagonists ( $10^{-4}$  M) on the electrically-evoked EPSP amplitude and duration began within 5 minutes of application and had generally reached a maximal effect within 30 minutes of application.

The decrease in the EPSP amplitude when the muscarinic antagonists are applied at the concentration of  $10^{-4}$  M, may be the result of the muscarinic cholinergic antagonists blocking nicotinic cholinergic receptors on BA1 which mediate the EPSPs.

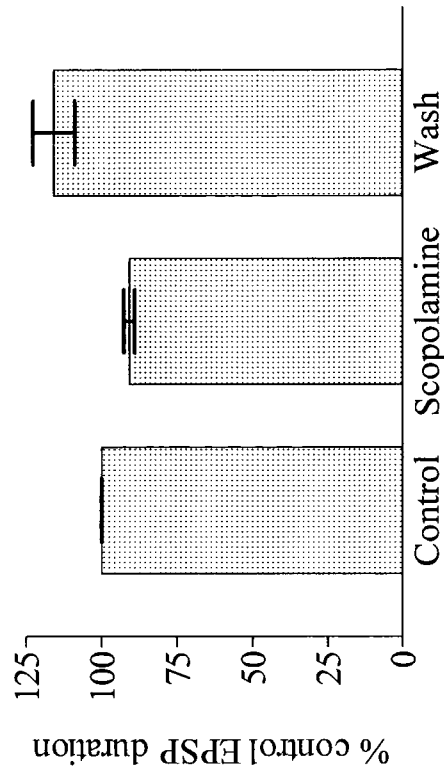
### **3.3.3. Effects of muscarinic cholinergic antagonists on ACh responses**

The increase in the electrically-evoked EPSP amplitude and duration induced by the muscarinic cholinergic antagonists at the concentration  $< 10^{-6}$  M may be due to the antagonists acting on presynaptic neurons and/or on the postsynaptic neuron BA1. To determine if the muscarinic antagonists cause BA1 to become more sensitive to ACh, their effects on the responses of BA1 to locally-applied ACh, were examined.

Under control conditions pressure application of brief pulses (100-500 ms) of ACh (100 mM) to the cell body of BA1 evoked depolarising responses with a mean amplitude of  $20.2 \pm 1.9$  mV (mean  $\pm$  SEM;  $n = 12$ ). Bath application of scopolamine at  $10^{-6}$  M (the same concentration which causes an increase in the EPSP amplitude) caused a reversible decrease in the ACh response amplitude (Figure 3.6A). A graphical representation of the data from several experiments ( $n = 8$ ) is shown in Figure 3.6B.



**Figure 3.5B. Maximum effect of Scopolamine ( $10^{-4}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of scopolamine and following wash ( $n = 14$ ). The amplitude is given as a percentage of the control amplitude.

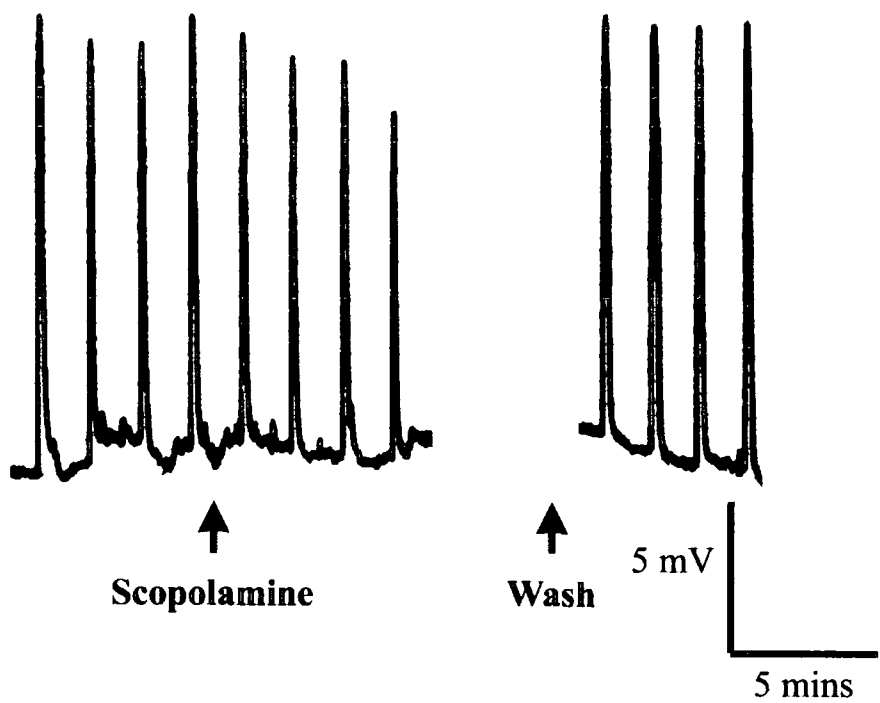


**Figure 3.5C. Maximum effect of Scopolamine ( $10^{-4}$  M) on EPSP duration.** Graphical representation of the pooled data from 14 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of scopolamine and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.

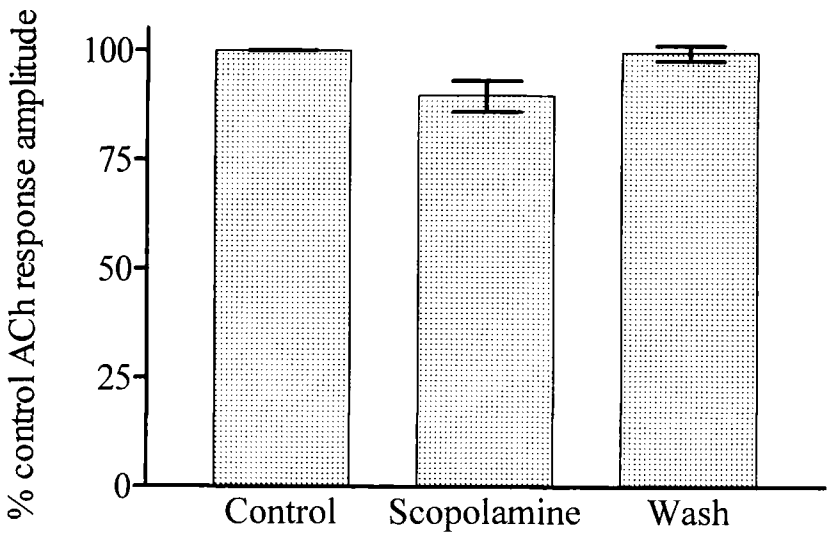
**Figure 3.6A. Effects of Scopolamine ( $10^{-6}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses (100-500 ms) of acetylcholine (ACh) (100 mM) onto the surface of the cell body of BA1, evoked depolarising responses in BA1. Following the application of scopolamine ( $10^{-6}$  M) the amplitude of the ACh responses decreased slightly. The decrease in the EPSP amplitude reversed after washing.

**Figure 3.6B. Maximum effect of Scopolamine ( $10^{-6}$  M) on ACh response amplitude.** Graphical representation of the pooled data from 8 experiments showing the mean amplitude ( $\pm$  SEM) of the ACh responses recorded from BA1 in the absence (control) and presence of scopolamine and following wash. The mean amplitude is given as a percentage of the control amplitude.

**Figure 3.6A. Effects of Scopolamine ( $10^{-6}$  M) on ACh responses recorded from BA1.**



**Figure 3.6B. Maximum effect of Scopolamine ( $10^{-6}$  M) on ACh response amplitude**





The ACh response amplitude decreased by a maximum mean percentage of 11.3 % (+ SEM 3.3; -SEM 3.8;  $p = 0.001$ ;  $n = 8$ ) and reversed to control levels after washing the preparation with drug free saline.

The effects of the scopolamine ( $10^{-6}$  M) on the ACh response amplitude began within 2 minutes of application and had generally reached a maximal effect within 5 minutes ( $n = 8$ ). The decrease in the ACh response amplitude reversed within approximately 5 minutes of washing the preparation with drug-free saline.

These results demonstrate that unlike the effect of scopolamine ( $10^{-6}$  M) on electrically-evoked EPSP amplitudes, the amplitude of the responses of BA1 to locally-applied ACh, was not increased. This indicates that the increase in the electrically-evoked EPSP amplitude following scopolamine ( $10^{-6}$  M) is not due to an increase in the sensitivity of BA1 to ACh but is due to the blockade of muscarinic receptors located presynaptically.

#### **3.3.4. Effects of a GABA antagonist on responses recorded from BA1**

The muscarinic cholinergic receptors located presynaptically at the fSR/BA1 synapse may be located on the fSR itself and down-regulate ACh release and/or on GABAergic interneurons in a feed-forward loop. In the latter case, activation of the muscarinic receptors on GABA interneurons, which receive inputs from collaterals of the fSR, would in turn stimulate the release of GABA onto the fSR terminals and inhibit further ACh release. A number of insect sensory afferents receive presynaptic inputs from GABAergic interneurons (locust cercal afferents, Boyan, 1988, Watson, 1990; locust campaniform sensilla afferents, Watson and England, 1991; locust hair

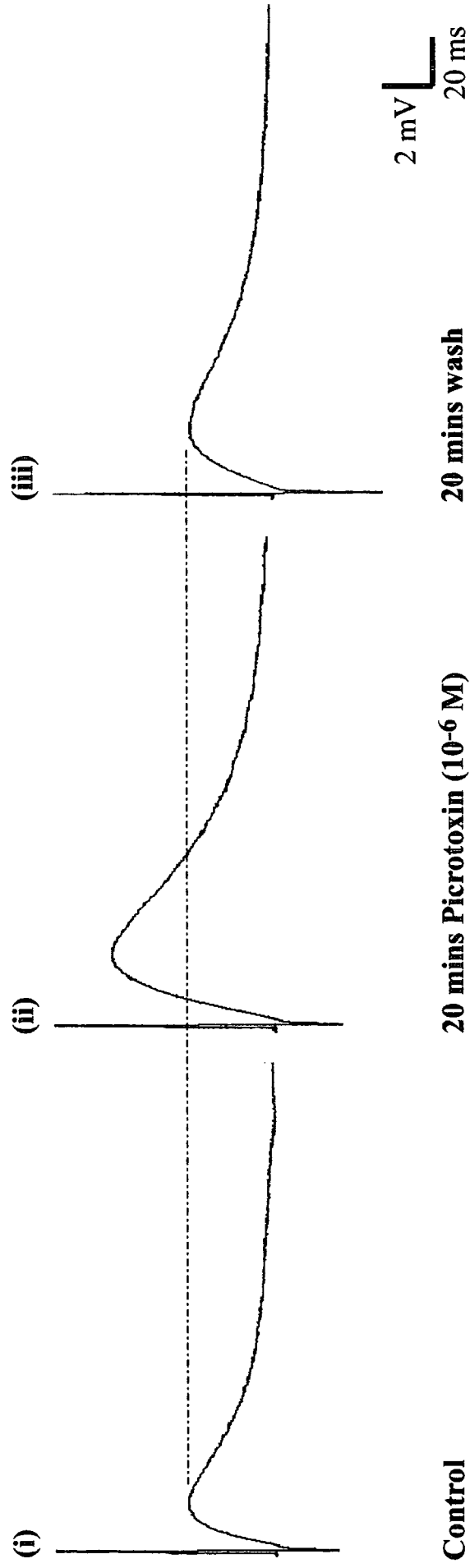
plate afferents, Watson et al., 1991; locust femoral chordotonal afferents, Burrows and Laurent, 1993, Watson et al., 1993; locust prosternal filiform afferents, Watson and Pflüger, 1994; bushcricket and cricket auditory organ afferents, Hardt and Watson, 1999; locust tegula afferents, Büschges and Wolf, 1999) so it is possible that GABAergic interneurons presynaptically inhibit the locust fSR. To investigate the possibility that a GABAergic feed-forward loop may be involved in modulating synaptic transmission at the fSR/BA1 synapse, the effects of the GABA antagonist picrotoxin on the electrically evoked EPSP (Figures 3.7, 3.8) and ACh response (Figure 3.9) recorded from BA1, were examined.

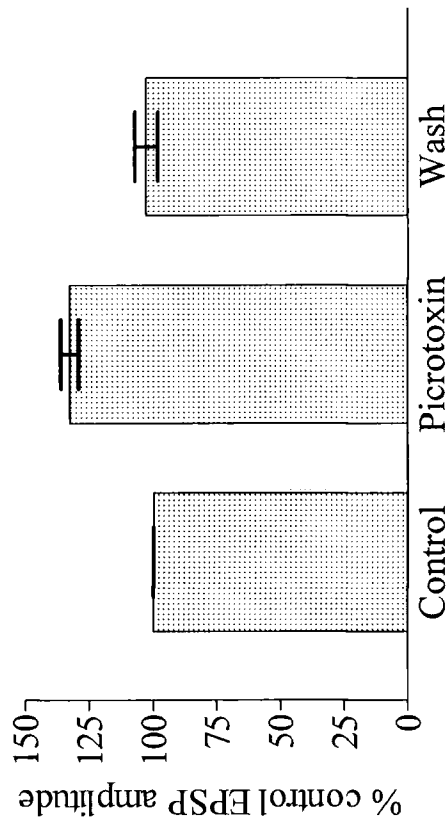
Under control conditions, electrical stimulation of the fSR evoked EPSPs in BA1 with a mean amplitude of  $4.6 \pm 0.4$  mV (mean  $\pm$  SEM;  $n = 11$ ) and a mean duration of  $120.7 \pm 8.5$  ms (mean  $\pm$  SEM;  $n = 11$ ). Bath application of picrotoxin ( $10^{-6}$  M) caused a reversible increase in the amplitude and duration of the electrically-evoked EPSP recorded from BA1 (Figure 3.7). Following the bath application of picrotoxin ( $10^{-6}$  M) the EPSP amplitude increased by a maximum mean percentage of 32.5 % (+ SEM 3.4; -SEM 3.6;  $p = 0.0004$ ;  $n = 11$ ) (Figure 3.8A) and the EPSP duration increased by a maximum mean percentage of 38.8 % (+ SEM 8.8; -SEM 9;  $p = 0.0013$ ;  $n = 11$ ) (Figure 3.8B). After washing with drug-free saline the EPSP amplitude and duration reversed.

The effects of picrotoxin ( $10^{-6}$  M) on the electrically-evoked EPSPs began within 5 minutes of application and had generally reached a maximal effect within 20 minutes of application. The EPSP amplitude and duration reversed within 20 minutes of washing the preparation with drug-free saline ( $n = 11$ ).

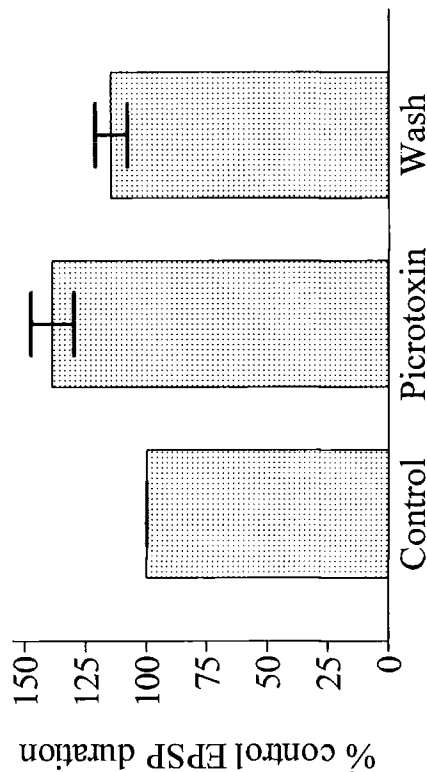
**Figure 3.7. Effects of Picrotoxin ( $10^{-6}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the selective electrical stimulation of the fSR axon. (ii) The GABA antagonist picrotoxin ( $10^{-6}$  M) caused a marked increase in the EPSP amplitude and duration which was reversible after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

**Figure 3.7. Effects of Picrotoxin ( $10^{-6}$  M) on electrically-evoked EPSPs.**





**Figure 3.8A. Maximum effect of Picrotoxin ( $10^{-6}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of picrotoxin and following wash ( $n = 11$ ). The amplitude is given as a percentage of the control amplitude.



**Figure 3.8B. Maximum effect of Picrotoxin ( $10^{-6}$  M) on EPSP duration.** Graphical representation of the pooled data from 11 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of picrotoxin and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.

Bath application of picrotoxin ( $10^{-6}$  M) caused the membrane potential of BA1 to fluctuate by  $2.4 \pm 1.1$  mV (mean  $\pm$  SEM) and the input resistance to change by  $0.6 \pm 0.5$  M $\Omega$  (mean  $\pm$  SEM). The small changes in the membrane potential ( $p = 0.08$ ) and the input resistance ( $p = 0.3$ ) are statistically insignificant. This indicates that the effects of picrotoxin ( $10^{-6}$  M) on electrically-evoked EPSPs are not due to changes in the membrane conductance of BA1, although it is possible that changes do occur beyond the recording site.

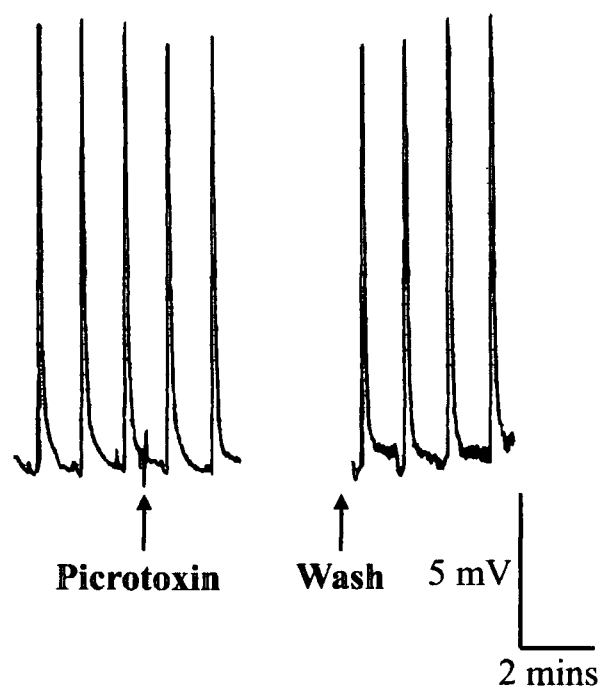
The reversible increase in the EPSP amplitude and duration following picrotoxin ( $10^{-6}$  M) application indicates that GABA receptors that are involved in modulating synaptic transmission between the fSR and BA1 are being blocked by the GABA antagonist. To eliminate the possibility that the increase in the EPSP amplitude is due to picrotoxin causing the postsynaptic neuron BA1 to become more sensitive to ACh, the effects of picrotoxin ( $10^{-6}$  M) on the responses of BA1 to locally applied ACh, were examined.

Under control conditions pressure application of brief pulses (100-500 ms) of ACh to the cell body of BA1 evoked depolarising responses with a mean amplitude of  $18.1 \pm 2$  mV (mean  $\pm$  SEM;  $n = 5$ ). In contrast to the effect of picrotoxin ( $10^{-6}$  M) on electrically-evoked EPSP amplitude (Figures 3.7, 3.8A), the amplitude of the ACh response following the bath application of picrotoxin ( $10^{-6}$  M) did not increase (Figure 3.9) In fact, picrotoxin ( $10^{-6}$  M) caused a reversible decrease in the ACh response amplitude (Figure 3.9A). The ACh response amplitude decreased by a maximum mean percentage of 7 % (+ SEM 2.1; - SEM 2.4; ;  $n = 5$ ) and reversed to approximately control levels after washing with drug-free saline (Figure 3.9B). The effects of

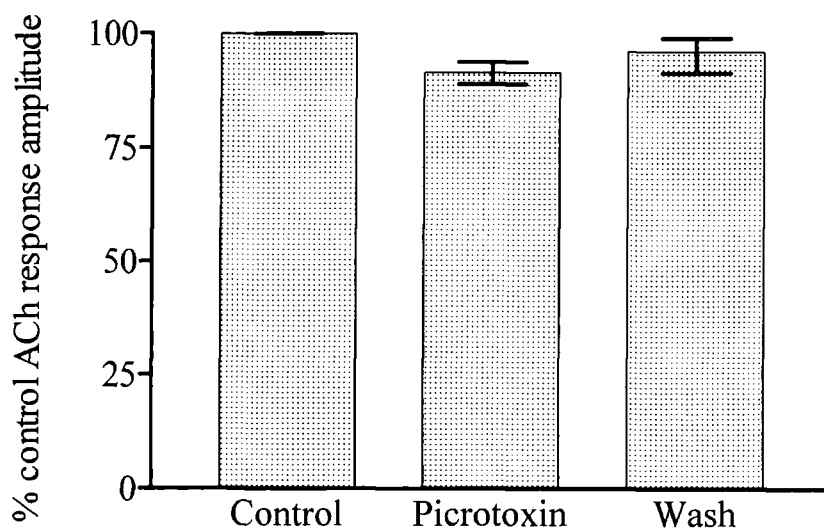
**Figure 3.9A. Effects of Picrotoxin ( $10^{-6}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses (100-500 ms) of acetylcholine (ACh) onto the cell body of BA1 resulted in depolarising responses in BA1. Following the application of picrotoxin ( $10^{-6}$  M) the amplitude of the ACh responses decreased slightly but reversed after washing.

**Figure 3.9B. Maximum effect of Picrotoxin ( $10^{-6}$  M) on ACh response amplitude.** Graphical representation of the pooled data from 5 experiments showing the amplitude of the ACh responses recorded from BA1 in the absence (control) and presence of picrotoxin and following wash. The mean ( $\pm$  SEM) amplitude is given as a percentage of the control amplitude.

**Figure 3.9A. Effects of Picrotoxin ( $10^{-6}$  M) on ACh responses recorded from BA1.**



**Figure 3.9B. Maximum effect of Picrotoxin ( $10^{-6}$  M) on ACh response amplitude**





picrotoxin ( $10^{-6}$  M) on the ACh response amplitude began within 2 minutes of application and had stabilised within 5 minutes. The decrease in the ACh response amplitude reversed within approximately 3 minutes of washing the preparation with drug-free saline .

The decrease in the ACh response amplitude shows that picrotoxin ( $10^{-6}$  M) does not cause BA1 to become more sensitive to ACh. The increase in the EPSP amplitude following picrotoxin ( $10^{-6}$  M) application is likely therefore, to be the result of inhibitory GABA receptors located presynaptically being blocked by the GABA antagonist picrotoxin.

### **3.3.5. Effects of scopolamine in a preparation preincubated with a GABA antagonist**

The results presented so far indicate that presynaptic muscarinic cholinergic receptors are involved in the modulation of the fSR/BA1 synapse. It has been suggested that they may be located on the fSR itself and act as autoreceptors and/or on a GABAergic interneuron in a feed-forward loop which when activated by the muscarinic receptors releases GABA. The GABA in turn may activate the inhibitory GABA receptors, which the results indicate are located presynaptically at the fSR/BA1 synapse. If GABAergic interneurons activated by muscarinic receptors are involved in the modulating the fSR/BA1 synapse, blockade of the GABA receptors with picrotoxin should prevent the increase in the EPSP amplitude normally caused by the muscarinic antagonists. If, on the other hand, down-regulation of synaptic transmission between the fSR and BA1 is mediated by autoreceptors, the GABA

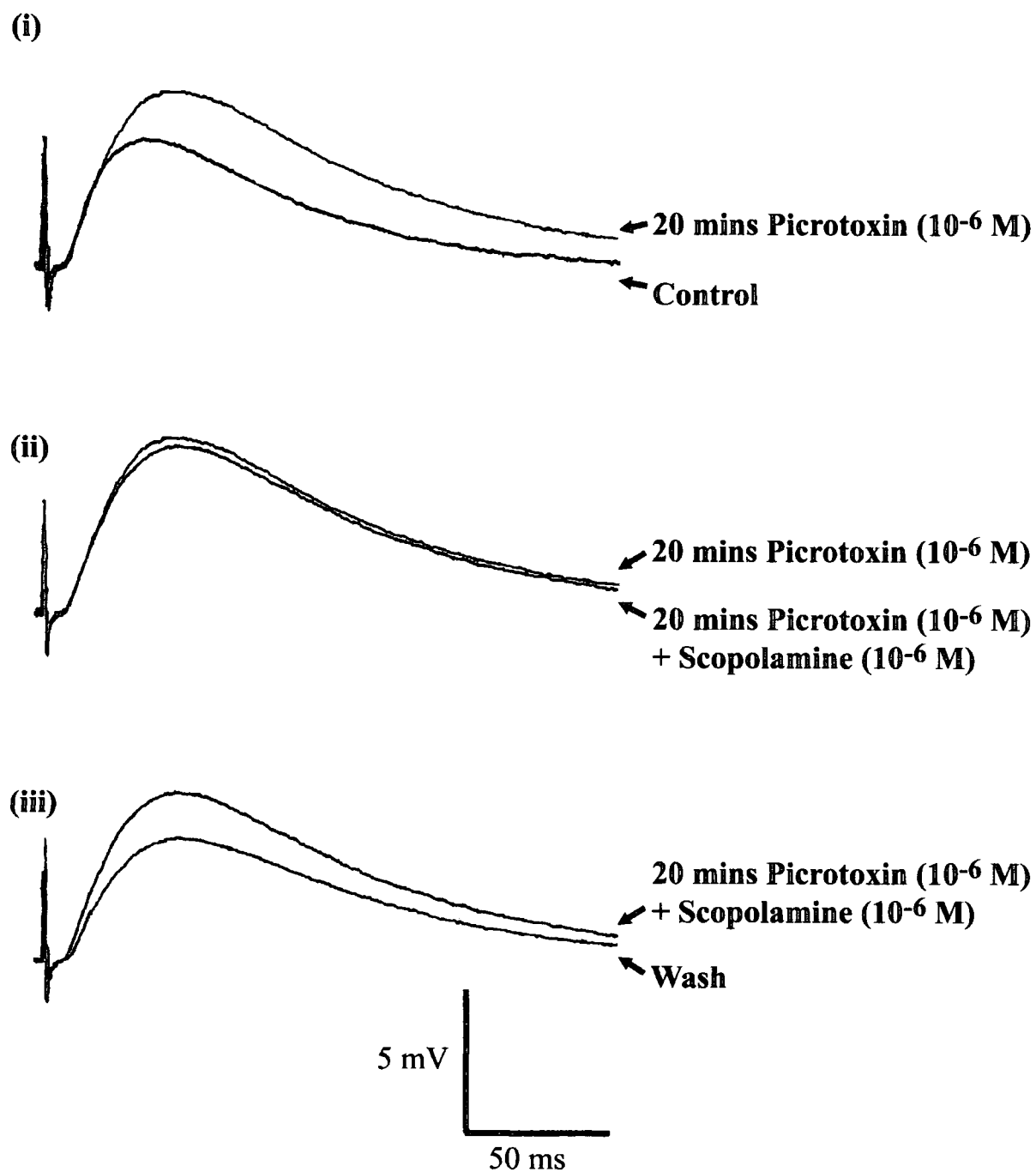
antagonist should not block the effects of muscarinic antagonists. The effects of scopolamine ( $10^{-6}$  M) in a preparation preincubated with picrotoxin ( $10^{-6}$  M) were therefore examined, to establish whether an inhibitory feed-forward loop is activated by the presynaptic muscarinic receptors involved in the down-regulation of ACh release (Figures 3.10, 3.11).

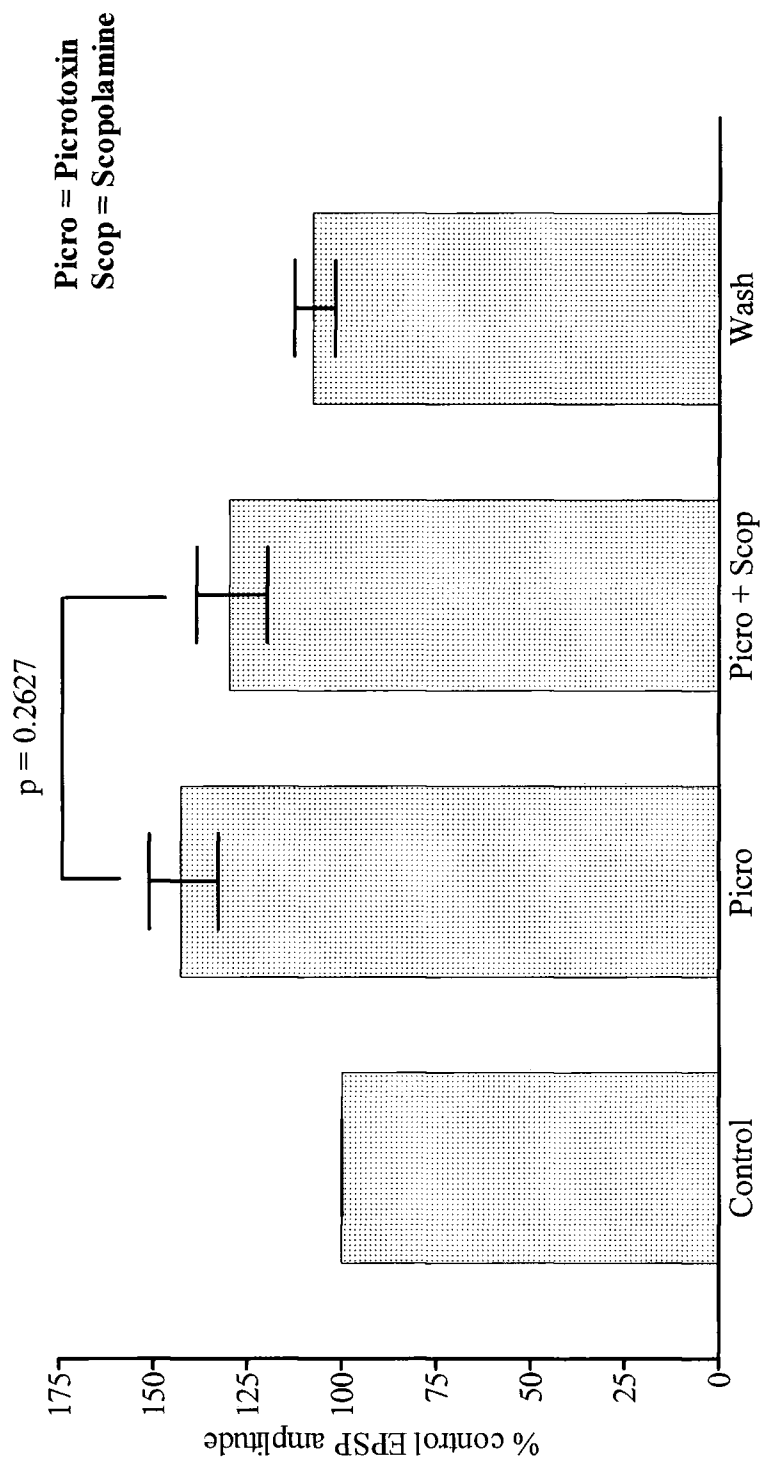
Figure 3.10 shows a sequential set of EPSP traces from one experiment in which a preparation was preincubated with picrotoxin ( $10^{-6}$  M) (Figure 3.10i) followed by bath application of scopolamine ( $10^{-6}$  M) (Figure 3.10ii). Preincubating the preparation with picrotoxin ( $10^{-6}$  M) caused a marked increase in the electrically-evoked EPSP amplitude (Figure 3.10i). Once the effects of picrotoxin on the EPSP amplitude had stabilised (after approximately 20 minutes) the muscarinic antagonist scopolamine ( $10^{-6}$  M) was subsequently applied to the same preparation (3.10ii). Bath application of scopolamine ( $10^{-6}$  M) caused the augmented EPSP amplitude to decrease by 12.9 % (+ SEM 8.8; - SEM 9.9;  $p = 0.2627$ ;  $n = 6$ ) (Figure 3.11). The EPSP amplitude began to decrease within 5 minutes of application and had stabilised within 20 minutes. The EPSP amplitude returned to control levels within 30 minutes of washing the preparation with drug-free saline (Figure 3.10iii).

Therefore, unlike the effect of scopolamine ( $10^{-6}$  M) on the EPSP amplitude in control experiments (Figures 3.1, 3.3), the amplitude of the EPSPs did not increase in preparations preincubated with picrotoxin. This indicates that the pathway involving the presynaptic muscarinic receptors is already made ineffectual by the GABA antagonist picrotoxin. This is consistent with the hypothesis that at least some of the presynaptic muscarinic receptors at the fSR/BA1 synapse are located on GABAergic interneurons.

**Figure 3.10. Effects of Scopolamine ( $10^{-6}$  M) on electrically-evoked EPSPs in a preparation preincubated with Picrotoxin ( $10^{-6}$  M).** (i) Preincubating the preparation with the GABA antagonist picrotoxin caused a marked increase in the amplitude of the excitatory postsynaptic potentials (EPSPs). (ii) Applying the muscarinic antagonist scopolamine ( $10^{-6}$  M) to the same preparation caused a slight reduction in the EPSP amplitude. It did not cause an increase in the EPSP amplitude seen in control experiments in the absence of picrotoxin. (iii) Perfusing the preparation with fresh saline caused the EPSP amplitude to return to control levels.

**Figure 3.10. Effects of Scopolamine ( $10^{-6}$  M) on electrically-evoked EPSPs in a preparation preincubated with Picrotoxin ( $10^{-6}$  M).**





**Figure 3.11. Effects of Scopolamine ( $10^{-6}$  M) on EPSP amplitude in the presence of Picrotoxin ( $10^{-6}$  M).** Graphical representation of pooled arc sine transformed data showing the mean ( $\pm$  SEM) amplitude of EPSP amplitudes ( $n = 6$ ). The electrically-evoked augmented EPSP amplitude recorded from BA1 in the presence of picrotoxin decreased slightly following scopolamine application. It did not cause an increase in the EPSP amplitude as seen in control experiments in the absence of picrotoxin. The P value was calculated using a two-tailed t-test.

#### 4. 4. DISCUSSION

The results from the electrophysiological experiments presented in this chapter confirm that presynaptic muscarinic cholinergic receptors are involved in modulating cholinergic synaptic transmission between the fSR and BA1 in the locust *Locusta migratoria*. The results also indicate that the fSR/BA1 synapse is modulated by GABAergic interneurons that inhibit ACh release from the presynaptic fSR terminals. The results from further experiments, which examined the effects of the muscarinic antagonist scopolamine in the presence of the GABA antagonist picrotoxin, are consistent with the hypothesis that at least some of the presynaptic muscarinic receptors are located on GABAergic interneurons.

##### 3.4.1. Modulatory presynaptic muscarinic cholinergic receptors

The results confirm that muscarinic cholinergic receptors are involved in modulating transmission at the fSR/BA1 synapse in the locust *Locusta migratoria*. Bath application of the general muscarinic antagonists scopolamine ( $10^{-6}$  M) and atropine ( $10^{-8}$ - $10^{-6}$  M) caused an increase in the amplitude and duration of electrically-evoked EPSPs recorded from BA1 (Figures 3.1 - 3.4). The increase in the EPSP amplitude was not due the antagonists increasing the sensitivity of the postsynaptic neuron BA1 to ACh, as the muscarinic antagonists did not cause an increase in BA1's responses to locally-applied ACh (Figure 3.6). This indicates that the increase in the EPSP amplitude was the result of the antagonists blocking presynaptic muscarinic receptors.

Presynaptic muscarinic receptors have been identified at the same synapse in the locust *Schistocerca gregaria* (Leitch and Pitman, 1995) and in other insect preparations (Hue et al., 1989; Trimmer and Weeks, 1989; Le Corrionc et al., 1991; Parker and Newland, 1995). As in our experiments, application of general muscarinic antagonists to these other insect afferent preparations possessing modulatory muscarinic receptors caused an increase in the EPSPs recorded from the postsynaptic neuron. At the cockroach cercal afferent/giant interneuron synapses, application of the muscarinic antagonist atropine ( $5 \times 10^{-7}$  M) caused a 89% increase in the amplitude of unitary EPSPs (Hue et al., 1989) and application of scopolamine methyl bromide ( $2 \times 10^{-7}$  M) enhanced EPSPs at the planta hair afferent/proleg retractor motoneuron synapses in the tobacco hornworm, *Manduca sexta*, by approximately 50% (Trimmer and Weeks, 1989). In this study the mean amplitude of EPSPs recorded from BA1 in *Locusta migratoria*, increased by 10.8% following the application of scopolamine ( $10^{-6}$  M), 22.2% following the application of atropine ( $10^{-8}$  M), and 23% following the application of atropine ( $10^{-8}$  M). At the same synapse in the locust *Schistocerca gregaria*, application of scopolamine ( $10^{-4}$  M) and atropine ( $10^{-4}$  M) resulted in the EPSP amplitude increasing by a mean percentage of 25.75 and 28.2%, respectively (Leitch and Pitman, 1995).

The differing magnitudes of effect the muscarinic antagonists have on the amplitude of EPSPs at different cholinergic synapses may be simply due to different experimental set-ups and procedures. It is possible, however, there may be more functional explanations. Firstly, muscarinic antagonists may have a greater effect on the cholinergic synapses which consist of a population of cholinergic sensory afferents converging on a single postsynaptic neuron than on the synapses consisting

of a postsynaptic neuron receiving inputs from solitary afferents; the combined effect of blocking the muscarinic receptors on a population of afferents may result in a greater increase in EPSP amplitude. At the fSR/BA1 synapse which is a single sensory neuron connecting with a motoneuron, muscarinic antagonists induced an increase in the EPSP amplitude of less than 30%, whereas at the other insect afferent synapses that are part of sensory arrays which converge upon one postsynaptic neuron, muscarinic antagonists caused an increase in the EPSP amplitude of more than 50%. Secondly, variations in the effects the muscarinic antagonists have on the postsynaptic responses in different insect preparations may account for the differing magnitudes of effect the antagonists have on EPSP amplitudes. For example, the blockade of postsynaptic receptors responsible for the postsynaptic responses, may reduce the increase in the EPSP amplitude produced through the blockade of the presynaptic muscarinic receptors. The muscarinic antagonists will block postsynaptic muscarinic receptors on BA1 which appear to be partly responsible for postsynaptic responses to ACh [scopolamine ( $10^{-6}$  M) reduces the amplitude of ACh responses recorded from BA1 (Figure 3.6)]. Thirdly, the variations in the magnitude of the effect mediated by muscarinic antagonists could be the result of the muscarinic receptors acting through different mechanisms. The muscarinic receptors could be autoreceptors and directly down-regulate ACh release from the presynaptic terminals of the afferents and/or they may be acting indirectly through inhibitory GABAergic interneurons as discussed previously.

The muscarinic receptors located presynaptically at the fSR/BA1 synapse in the locust *Locusta migratoria* have not been characterised in this study using antagonists selective for certain muscarinic receptor subtypes (Table 3.1.). It is likely though,



that they are M<sub>2</sub> muscarinic receptors, as the presynaptic muscarinic receptors characterised at the same synapse in *Schistocerca gregaria* (Leitch and Pitman, 1995) and in other insect preparations are M<sub>2</sub> muscarinic receptors (Knipper and Breer, 1988; Le Corrionc et al., 1991).

The reversible decrease in the EPSP amplitude following the application of high ( $10^{-4}$  M) concentrations of the general muscarinic antagonists observed in this study (Figure 3.5) is likely to be due to the blockade of postsynaptic cholinergic receptors on BA1 responsible for mediating the EPSPs. The majority of the postsynaptic cholinergic receptors on BA1 responsible for mediating the EPSPs are nicotinic, as the application of nicotinic antagonists markedly suppresses the EPSPs recorded from BA1 (Leitch and Pitman, 1995). The decrease in the EPSP amplitude caused by high concentrations of muscarinic antagonists is consistent with the fact that muscarinic antagonists are known to block insect nicotinic receptors at high concentrations (Shankland et al., 1971; Schmidt-Nielsen et al., 1977; David and Sattelle, 1984).

This concentration-dependent effect on the EPSP amplitude has also been observed at other insect cholinergic synapses (Hue et al., 1989; Trimmer and Weeks, 1989). At the cockroach cercal afferent/giant interneuron synapses application of atropine ( $10^{-8}$ - $10^{-6}$  M) causes an increase in the EPSP amplitude but atropine ( $10^{-5}$  M) causes a decrease. At the *Manduca* hair afferent/proleg retractor motoneuron synapses application of scopolamine methyl bromide ( $2 \times 10^{-7}$  M) causes an increase in the EPSP amplitude but concentrations higher than  $10^{-4}$  M cause a decrease (Trimmer and Weeks, 1989). These results show, that although the muscarinic antagonists are selective they are not specific for muscarinic receptors at high concentrations and reminds us that caution must be taken in assuming that a pharmacological agent is

acting at a certain type of receptor. However, low concentrations of the muscarinic antagonists do appear to be specific for muscarinic receptors, so the results do indicate that the fSR/BA1 synapse is modulated by presynaptic muscarinic receptors.

### **3.4.2. The role of GABAergic interneurons**

The results presented in this chapter indicate that the fSR/BA1 synapse in the locust *Locusta migratoria* is also presynaptically inhibited by GABA. Bath application of the GABA antagonist picrotoxin, which has been successfully used to block the effects of applied or synaptically released GABA on other insect neurons (Lees et al., 1987; Watson and Burrows, 1987; Benson, 1988; Sattelle et al., 1988; Burrows and Laurent, 1993), caused an increase in the EPSP amplitude recorded from BA1 (Figures 3.7, 3.8). The increase in the EPSP amplitude could either be due to picrotoxin blocking presynaptic GABA receptors normally involved in inhibiting ACh release from the fSR terminals, or due to picrotoxin increasing the sensitivity of the postsynaptic neuron BA1 to ACh. As picrotoxin did not cause an increase in BA1's responses to locally-applied ACh (Figure 3.9), it was concluded that the increase in the EPSP amplitude was the result of picrotoxin blocking presynaptic GABA receptors. This conclusion, however, is based upon the assumption that the receptors on the cell body of BA1, to which the ACh is locally-applied, are the same as those on the postsynaptic membrane of BA1 within the neuropil. There is some evidence to suggest that GABA receptors on the cell body of insect neurons may be different to insect synaptic GABA receptors (see Benson, 1993; Dubreil et al., 1994). This suggestion, however, is based upon differences in the sensitivity of somal and synaptic GABA receptors to the GABA antagonist bicuculline rather than picrotoxin.

GABAergic neurons are very important in insect flight and evidence of this has been demonstrated in a recent ICC study (Witten and Truman, 1998), which has examined the distribution of GABA-IR neurons in the thoracic ganglia of nine orders of insects. The number of GABA-IR neurons per ganglion (4000-5000) in the advanced orders of insects with well co-ordinated flight such as *Manduca* and *Drosophila*, is much higher than the number of GABA-IR neurons per ganglion (1500) in the apterygote orders, which are flightless insects (Witten and Truman, 1998). In the locust flight system, the existence of GABAergic interneurons is well-documented (Robertson and Pearson, 1983; Pearson and Robertson, 1987; Reye and Pearson, 1987; Robertson and Wisniowski, 1988) and eight intersegmental interneurons that are GABA-IR have been identified (301, 302, 501, 507, 511, 513, 520, 538) (Robertson and Wisniowski, 1988).

The GABAergic interneurons implicated in the present study are probably stimulated by collaterals of the fSR. The fSR is reported to connect with at least 21 flight interneurons (Reye and Pearson, 1987) and of these, 5 are known to be GABA-IR (Robertson and Wisniowski, 1988), making these the most likely candidates for the inhibition of the fSR terminals. However, it is also likely that the GABAergic interneurons which inhibit the fSR are activated by at least one other neuron involved in locust flight.

The reason the fSR is presynaptically inhibited by GABA is undetermined. In other invertebrate preparations, presynaptic inhibition of primary afferents protects the sensory pathway from synaptic depression (Kirk and Wine, 1984; Bryan and Krasne, 1977; Levine and Murphey, 1980), sharpens the directional sensitivity of the sensory

inputs (Wiese et al., 1976; Levine and Murphey, 1980) and provides a mechanism for controlling the gain among afferents of the same organ (Burrows and Laurent, 1993; Burrows and Matheson, 1994).

Recently, it has been reported that a wing proprioceptor, the tegula, is presynaptically inhibited (Büschges and Wolf, 1999). Primary afferent depolarisations (PADs), which are associated with presynaptic inhibition, have been recorded from the terminal arborizations of the tegula. The PADs are inhibited by the GABA antagonist picrotoxin, indicating that they are mediated by GABA. The function of the presynaptic inhibition of the tegula appears to be to prevent the wing from being elevated further during the elevator phase of the wing cycle. A reduction in the amplitude of the EPSPs recorded from the elevator motoneurons when the wing is elevated is thought to be due to the presynaptic inhibition of the tegula (Büschges and Wolf, 1999), which connects with the elevator motoneurons (Burrows, 1976). The tegula and the fSR are both wing proprioceptors but with opposite actions; the tegula is activated on the wing down-stroke and excites elevator motoneurons whereas, the fSR is activated on the wing up-stroke and excites depressor motoneurons. It is possible therefore, that the fSR is presynaptically inhibited to reduce the amplitude of EPSPs in the depressor motoneurons when the wing is depressed, to prevent it from being depressed further. This is a very simplistic explanation for the presynaptic inhibition of the fSR however and there is probably more than one reason.

For example, the GABAergic interneurons implicated in this study may play a role in integrating sensory information about different modalities into the flight motor program. The output of the fSR is known to raise the frequency of the flight motor

pattern, entrain it to the frequency of stimulation, and reset the wing rhythm when stimulated at the correct phase in the wing beat cycle (Pearson et al., 1983; Reye and Pearson, 1988) and the depressor motoneurons it connects with control the pronation of the flight as well as wing depression. Therefore, presynaptic inhibition of the fSR/BA1 synapse by GABAergic interneurons may allow fine adjustments to be made to the flight motor pattern, which is essential to control and maintain flight.

### **3.4.3. GABA interneurons activated via muscarinic receptors**

The results presented in this chapter indicate that the fSR/BA1 synapse is presynaptically modulated by muscarinic receptors and GABAergic interneurons. Other insect cholinergic synapses that are also presynaptically inhibited by muscarinic receptors and GABAergic interneurons include the cockroach cercal afferent/giant interneuron synapses (Hue and Callec, 1983, Hue et al., 1989, Le Corrionc et al., 1991) and the locust campaniform sensilla afferents/ FETi motoneuron synapses (Watson and England, 1991, Parker and Newland, 1995). It has generally been assumed that presynaptic muscarinic receptors are located on the terminals of the sensory afferent and act as autoreceptors but it is possible that they may also be located on inhibitory GABAergic interneurons. The results presented in this chapter indicate that at least some of the presynaptic muscarinic receptors at the fSR/BA1 synapse are located on GABAergic interneurons. Bath application of the muscarinic antagonist scopolamine to preparations preincubated with the GABA antagonist picrotoxin did not cause an increase in the EPSP amplitude normally observed in control experiments in the absence of picrotoxin (Figures 3.10, 3.11). This indicates that at least some of the muscarinic receptors are located on the GABAergic interneurons, which are rendered

ineffectual with the picrotoxin. It is not possible to conclude, however, that there are no muscarinic receptors located on the presynaptic terminals of the fSR.

Indirect evidence that presynaptic muscarinic receptors may act indirectly through GABAergic interneurons has been reported in a study at another insect cholinergic synapse (Le Corrionc et al., 1991). It was suggested that the GABAergic interneurons which presynaptically modulate the cockroach cercal afferents are partly-activated by muscarinic agonists, as preincubating the cercal afferent/giant interneuron preparation with picrotoxin ( $10^{-4}$  M) reduced the decrease in the amplitude of the EPSPs normally observed with muscarinic agonists.

In the vertebrate CNS, evidence that muscarinic receptors are located on GABAergic neurons has been demonstrated in ICC and electrophysiological studies. An ICC study has revealed that some GABA-IR terminals in the lateral posterior nucleus of the cat thalamus also stain for  $M_2$  muscarinic receptors, indicating that the muscarinic receptors control GABA release (Carden and Bickford, 1999). Muscarinic receptors also control GABA release from inhibitory neurons in the rat substantia nigra and substantia gelatinosa and from cerebral cortical GABA neurons (Hashimoto et al., 1994; Kayadjanian et al., 1994; Baba et al., 1998). Due to the complexity of the vertebrate CNS however, there has been a paucity of studies which have identified a sensory afferent that is presynaptically inhibited by GABAergic interneurons activated by muscarinic receptors.

Therefore, direct evidence of presynaptic muscarinic receptors acting indirectly through GABAergic interneurons to presynaptically inhibit a sensory afferent has not been reported previously.

## **Chapter 4. GABA-immunoreactivity in processes presynaptic to the fSR**

### **4.1. INTRODUCTION**

Electrophysiological studies can demonstrate that a neuron (e.g. an afferent) may be affected by applied or synaptically released neurotransmitters, but EM ICC studies can determine if the afferent receives direct synaptic inputs from neurons containing a particular neurotransmitter. The neurotransmitter present in a neuron can be identified by labelling the tissue with a primary antibody raised against the neurotransmitter.

Primary antibodies raised against the inhibitory neurotransmitter GABA (Segula et al., 1984) have been used to reveal the widespread distribution of GABA-immunoreactive (GABA-IR) neurons in the CNS of locust (Watson, 1986; Watson and Pflüger, 1987; O'Dell and Watkins, 1988), honey bee (Schäfer and Bicker, 1986), tobacco hornworm moth (Homborg et al., 1987), crayfish (Mulloney and Hall, 1990), and earthworm (Telkes et al., 1996). In the locust thoracic ganglia GABA-immunoreactivity is present in approximately 250 cell bodies in each of the thoracic ganglia and all the neuropil regions of the ganglia, except the anterior ventral association centre, are heavily labelled (Watson, 1986). Individual neurons in the locust CNS that have been identified as GABA-IR include: three common inhibitor motoneurons (Watson, 1986); one class of spiking local interneurons which make direct contact with motoneurons (Watson and Burrows, 1987); and certain intersegmental interneurons (Burrows et al., 1988) which include some of the interneurons involved in controlling flight (Robertson and Wisniowski, 1988).

The neurons that are immunoreactive for GABA contain vesicles that are morphologically unique (Watson, 1988). The vesicles in GABA-IR processes in the locust CNS are predominantly small, pleomorphic and agranular although some GABA-IR processes also contain a few large granular vesicles (Watson, 1988; Watson et al., 1991, 1993; Watson and Pflüger, 1994; Pflüger and Watson, 1995). It was reported that all processes in the locust CNS that contain small, pleomorphic, agranular vesicles are GABA-IR (Watson, 1988). However, the shape of the vesicles also depends to some extent on the tonicity of the fixative used (Tisdale and Nakajima, 1976). For example, pleomorphic vesicles in GABA-IR processes in the crayfish become round in hyperosmotic solutions (Tisdale and Nakajima, 1976). It is unwise therefore, to decide a neuron contains a certain neurotransmitter on the basis of the morphology of its vesicles alone, which demonstrates the importance of using primary antibodies to identify the neurotransmitter content of a neuron.

EM ICC studies using primary antibodies raised against GABA have shown that a number of sensory afferents and other neurons (cercal afferents, Watson, 1990; campaniform sensilla, Watson and England, 1991; hair plate afferents, Watson et al., 1991; FeCO afferents, Watson et al., 1993; prosternal filiform afferents, Watson and Pflüger, 1994; dorsal unpaired median neurons, Pflüger and Watson, 1995) receive direct synaptic inputs from GABA-IR processes. This ultrastructural evidence is supported by the results from electrophysiological studies (Boyan, 1988; Burrows and Laurent, 1993) and has led to the suggestion that GABA is the main neurotransmitter involved in presynaptic inhibition (Watson, 1992a). Electrophysiological experiments have shown that cercal filiform hair afferents and FeCO afferents, which both receive synaptic inputs from GABA-IR processes (Watson, 1990; Watson et al., 1993), also



receive primary afferent depolarisations (PADs) (Boyan, 1988; Burrows and Laurent, 1993). These PADs reduce the amplitudes of spikes in the afferents. The PADs received by the cercal filiform hair afferents come indirectly from the activation of unidentified afferents (Boyan, 1988) whereas the PADs received by the femoral chordotonal organ afferents are generated as a result of spikes in other afferents within the same organ (Burrows and Laurent, 1993). The femoral chordotonal organ afferents do not synapse directly with each other however, but are interposed by interneurons. The interneurons are thought to be GABAergic as the PADs in the chordotonal afferents can be mimicked by GABA application and blocked by picrotoxin (Burrows and Laurent, 1993).

Analysis of the number and type of inputs to insect sensory afferents reveals that, in addition to the synaptic inputs from GABA-IR processes, a significant proportion of inputs from processes are not immunoreactive to GABA (Watson, 1990; Watson and England, 1991; Watson et al., 1991, 1993; Watson and Pflüger, 1994). This indicates that the insect sensory afferents may be presynaptically modulated by other neurotransmitters. EM ICC studies on the locust hair plate afferents (Watson et al., 1991) and the prosternal filiform afferents (Watson and Pflüger, 1994), revealed that some of the non-GABA-IR processes presynaptic to the afferents are in fact immunoreactive for glutamate.

Glutamate is a major excitatory neurotransmitter in the vertebrate CNS but ICC (Bicker et al., 1988; Watson, 1988; Watson and Seymour-Laurent, 1993) and physiological studies (Sombati and Hoyle, 1984c; Wafford and Sattelle, 1989; Dubas, 1990, 1991; Parker, 1994; Pearlstein et al., 1994; Washio, 1994) indicate it also plays an important

role in the invertebrate CNS. Three hundred and sixty to four hundred glutamate-immunoreactive (glutamate-IR) cell bodies have been identified in the prothoracic and mesothoracic locust ganglia and about 600 in the metathoracic ganglion (Watson and Seymour-Laurent, 1993). The position and structure of many of the glutamate-IR cell bodies revealed by ICC, corresponds with the position and structure of known motoneurons in the locust thoracic ganglia (Watson and Seymour-Laurent, 1993). For example, glutamate-immunoreactivity has been found in identified locust motoneurons, such as the slow extensor tibiae motoneuron (SETi) and the fast extensor tibiae motoneuron (FETi) (Bicker et al., 1988). Some interneurons in the insect CNS are also thought to be glutamate-IR (Watson and Seymour-Laurent, 1993). The structure and position of a group of ascending intersegmental interneurons called the 404 interneurons, which are involved in initiating and maintaining locust flight (Pearson et al., 1985), matches the structure and position of a bundle of glutamatergic neurons revealed in an ICC study by Watson and Seymour-Laurent (1993).

The presence of glutamate-immunoreactivity in the neurons mentioned above does not necessarily prove glutamate is released as a neurotransmitter from these neurons. There is evidence however, that glutamate is released from locust motoneurons as an excitatory neurotransmitter at invertebrate neuromuscular synapses (Usherwood et al., 1968; Cull-Candy, 1976). Additionally, glutamate is reported to be released from invertebrate motoneurons at central synapses (Sombati and Hoyle, 1984c; Parker, 1994; Pearlstein et al., 1994). The locust FETi, which is glutamate-IR (Bicker et al., 1988), makes excitatory connections with its antagonists, the flexor tibiae motoneurons. FETi-evoked EPSPs recorded from the flexor tibiae motoneurons, are abolished following bath application of a glutamate antagonist (Parker, 1994), indicating synaptic

transmission between the motoneurons is glutamatergic. In addition, injection of glutamate into specific neuropil regions in the metathoracic ganglion which contain the FETi and flexor motoneurons excite both the FETi and flexor motoneurons (Sombati and Hoyle, 1984c; Parker 1994). Pressure ejection of glutamate, however, onto the cell bodies of the FETi and the flexor motoneuron PFF1 on the surface of the ganglion, generally causes the cells to hyperpolarise (Parker, 1994).

Glutamate has been reported to evoke both depolarising and hyperpolarising responses in other invertebrate motoneurons (Wafford and Sattelle, 1989; Dubas, 1990, 1991; Washio, 1994). Thirty-eight to forty-seven percent of locust flight motoneurons tested (Dubas, 1990, 1991) depolarise following glutamate application while another, 40-46% of locust flight motoneurons tested hyperpolarise following glutamate application (Dubas, 1990, 1991). Glutamate also causes the cockroach Df motoneuron to hyperpolarise (Wafford and Sattelle, 1989) and the cockroach dorsal unpaired median (DUM) neurons to hyperpolarise or depolarise depending on the resting potential of the neurons (Washio, 1994). In addition to hyperpolarising or depolarising responses, some of the flight motoneurons tested produce biphasic responses following the application of glutamate (Dubas, 1991). Biphasic changes in the membrane potential in response to glutamate, which have also been recorded from the locust extensor tibiae muscle (Cull-Candy, 1976) and dissociated neuronal somata from the locust CNS (Giles and Usherwood, 1985), indicates that two different types of glutamate receptors are being activated (see Usherwood, 1994).

Evidence of two different types of receptors present on the same neuron has also been shown in other studies (Wafford and Sattelle, 1989). For example, neurons such as the

cockroach motoneuron Df depolarises in response to the glutamate receptor agonists, kainate and quisqualate and hyperpolarises in response to glutamate (Wafford and Sattelle, 1989). Receptors which cause the neuron to depolarise appear to gate cationic channels (Wafford and Sattelle, 1989; Ultsch et al., 1992), whereas, receptors which cause the neuron to hyperpolarise appear to gate chloride channels (Wafford and Sattelle, 1989; Dubas, 1990; Pearlstein et al., 1994; Washio, 1994). The hyperpolarisations induced by glutamate in the cockroach Df motoneuron (Wafford and Sattelle, 1989) and DUM neurons (Washio, 1994), in the locust flight motoneurons (Dubas, 1990), and in a crayfish motoneuron (Pearlstein et al., 1994) are all chloride dependent and are blocked by the chloride channel blocker, picrotoxin (Wafford and Sattelle, 1989; Pearlstein et al., 1994; Washio, 1994). The invertebrate glutamate-gated chloride channels which mediate the hyperpolarisations are interesting because equivalents have not been found in vertebrates. Recent molecular biology studies (see Cully et al., 1996) on the invertebrate glutamate-gated chloride channels have revealed that the glutamate-gated chloride channels belong to the same family of receptors as GABA and glycine receptors. Although invertebrate GABA and glutamate receptors do belong to the same family and mediate similar effects in the same neuron (Wafford and Sattelle, 1989; Dubas, 1991) they do not appear to mediate their effects via the same mechanism (Wafford and Sattelle, 1989; Dubas, 1991). The reversal potential of the neurons responses to glutamate and GABA are different (Dubas, 1991), desensitisation to one neurotransmitter does not affect the response of the neuron to the other neurotransmitter (Dubas, 1991), and antagonists selective for one type of receptor do not block the other receptor type (Wafford and Sattelle, 1989).

The locust prosternal filiform afferents and hair plate afferents are also likely to possess both glutamate and GABA receptors. The EM ICC studies mentioned previously (Watson et al., 1991; Watson and Pflüger, 1994) reported that the afferents receive inputs from both GABA-IR and glutamate-IR processes, indicating that the afferents are presynaptically modulated by GABA and glutamate. The results from Chapter 3 indicate that the sensory afferent, fSR may be presynaptically modulated by GABA. The amplitude of EPSPs evoked in the depressor motoneuron BA1, by the electrical stimulation of the fSR, increased in the presence of the GABA antagonist picrotoxin, indicating that GABA receptors that are normally involved in inhibiting the fSR were being blocked (Judge and Leitch, 1999c). The aims of the experiments conducted in this chapter were; a) to establish if the fSR does receive direct synaptic inputs from GABA-IR processes which could modulate synaptic efficacy at the fSR/BA1 synapse, b) to determine what proportion of inputs to the fSR are GABA-IR, and thus assess the relative importance of GABAergic modulation at this synapse, c) to analyse the distribution of GABA-IR inputs relative to other non-GABA-IR inputs and outputs to determine whether GABA-IR inputs, and hence the sites for mediating inhibition, are restricted to particular sites on the fSR and d) to establish if the fSR also receives inputs from glutamate-IR processes. The majority of this work has now been published (Judge and Leitch, 1999b).

## **4.2. MATERIALS AND METHODS**

### **4.2.1. Intracellular staining and histology for light microscopy**

The gross morphology of the fSR was revealed by back-filling the fSR with cobalt chloride *in vitro* and examining both thoracic nerve cord wholemounts and transverse sections through the mesothoracic ganglion at the light microscope level. This has been described in detail in Chapter 2.3.

The central projections of the fSR were drawn in detail with the aid of a camera lucida (Figure 4.1). The levels at which the sections selected for display (Figure 4.1C-F) were taken are indicated by the thick horizontal lines in Figure 4.1A. The nomenclature of the commissures, tracts and neuropils is that used by Tyrer and Gregory (1982) and Pflüger et al (1988). Abbreviations are shown in Table 4.1.

### **4.2.2. Electron microscopy**

The presence and distribution of GABA-IR processes presynaptic to the fSR, was established by examining double-labelled ultrathin sections at the EM level. In total, 5 locust mesothoracic ganglia were prepared and examined. The fSR was labelled by back-filling the fSR with HRP *in vivo* and incubating the mesothoracic ganglia containing HRP-filled fSR with DAB to produce an electron dense DAB-HRP reaction product in the fSR branches. This method is described in detail in Chapter 2.4.

**Table 4.1. Abbreviations of commissures, tracts, and neuropils used in the text and figures.**

---

aLAC	Anterior lateral association centre
DCI-IV	Dorsal commissures I-IV
DIT	Dorsal intermediate tract
DMT	Dorsal median tract
LDT	Lateral dorsal tract
SMC	Supramedian commissure
VCII	Ventral commissure II
VIT	Ventral intermediate tract

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#### **4.2.3. Immunocytochemistry with an anti-GABA antibody**

Ultrathin sections were taken through the mesothoracic ganglia containing HRP-filled fSR at each of the levels shown in Figure 4.1. The ultrathin sections were incubated in rabbit anti-GABA antiserum (Sigma, Poole, U.K.) diluted 1:500. The antibody had been obtained from rabbits after immunisation with a GABA-glutaraldehyde-BSA conjugate (Segula et al., 1984) and had been tested at doubling dilutions from 1:50 to 1:32,000; the optimal staining was achieved with dilutions from 1:100-1:500 (Leitch and Laurent, 1996). The method used to immunolabel sections for EM has been described in detail in Chapter 2.5.

A number of positive and negative controls were performed to test the specificity of the immunolabelling. The anti-GABA antiserum used in this study bound specifically to axons in sections of nerve 5A of the metathoracic ganglion which are known to be GABAergic but did not label locust nervous tissue when it had been preabsorbed with BSA/GABA conjugate (Leitch and Laurent, 1993). Other negative controls included; a) replacing the anti-GABA antiserum with non-immune serum (Leitch and Laurent, 1996) and b) omitting the anti-GABA antiserum and replacing it with buffer only. None of the negative controls showed immunolabelling.

Examination of the sections labelled with anti-GABA antiserum revealed immunolabelled processes, indicated by a concentrated covering of gold beads. A paired t-test (Table 4.2) was used to assess if the density of gold beads over immunolabelled processes was significantly different to background levels (Chapter 2.5).



**Table 4.2. Mean density of gold particles over GABA-immunoreactive and non-immunoreactive profiles.**

Profile	Label density (particles/ $\mu\text{m}^2$ )
GABA-immunoreactive	$23.3 \pm 15.9$
Non-GABA-immunoreactive	$2.3 \pm 1.3$

$P < 0.0001$

The mean and SEM ( $n = 40$ ) of the gold particle density over GABA-immunoreactive profiles (immunolabelled) and adjacent non-immunoreactive profiles (covered in background levels) are given. A paired t-test was used to assess the significance of the gold particle density distribution over GABA-immunoreactive and non-immunoreactive profiles. The probability for the null hypothesis that the distribution of particles is random is also shown.

#### 4.2.4. Immunocytochemistry with an anti-glutamate antibody

To determine if glutamate-IR processes are presynaptic to the fSR, ultrathin sections taken from mesothoracic ganglia containing HRP-filled fSR were incubated with rabbit anti-glutamate antiserum (batch Glu607, donated from J. Storm-Mathisen, University of Oslo, Norway) diluted 1:1000. The antibody had been obtained from rabbits after immunisation with a glutamate-glutaraldehyde-BSA conjugate. The method used to immunolabel sections for EM has been described in detail in Chapter 2.5.

The specificity of the immunolabelling was tested with a number of positive and negative controls. The anti-glutamate antiserum bound specifically to known glutamatergic motoneurons in the locust (Bicker et al., 1988) but did not label locust nervous tissue when it had been preabsorbed with glutamate/BSA conjugate (Watson et al., 1991). Replacing the anti-glutamate antiserum with buffer only also prevented any immunolabelling.

Sections incubated in the anti-glutamate antibody contained immunolabelled processes, indicated by a concentrated covering of gold beads. A paired t-test (Table 4.3) was used to determine if the density of gold beads over immunolabelled processes was significantly different to background levels (Chapter 2.5).

Anti-glutamate antiserum has been previously reported to label GABA-IR processes (Watson et al., 1991). Therefore, serial sections labelled alternatively with anti-glutamate antibody and anti-GABA antibody were also examined to determine if the

**Table 4.3. Mean density of gold particles over glutamate-immunoreactive and non-immunoreactive profiles.**

Profile	Label density (particles/ $\mu\text{m}^2$ )
Glutamate-immunoreactive	$40.67 \pm 3.44$
Non-immunoreactive	$2.69 \pm 0.87$

$P < 0.0001$

The mean and SEM ( $n = 15$ ) of the gold particle density over glutamate-immunoreactive profiles (immunolabelled) and adjacent non-immunoreactive profiles (covered in background levels) are given. A paired t-test was used to assess the significance of the gold particle density distribution over glutamate-immunoreactive profiles and non-immunoreactive profiles. The probability for the null hypothesis that the distribution of the particles is random is also shown.

**Table 4.4. Mean density of gold particles over identified GABA-immunoreactive profiles in serial sections labelled with anti-GABA antibody and anti-glutamate antibody.**

Antibody	Label density (particles/ $\mu\text{m}^2$ )
Anti-GABA	$41 \pm 4.18$
Anti-glutamate	$2.55 \pm 0.85$

$P < 0.0001$

The mean and SEM ( $n = 11$ ) of the gold particle density over GABA-immunoreactive profiles which have been identified in serial sections labelled with anti-GABA antibody and anti-glutamate antibody are given. A paired t-test was used to assess the significance of the gold density distribution over GABA-immunoreactive profiles in serial sections labelled with anti-GABA antibody and anti-glutamate antibody. The probability for the null hypothesis that the distribution of the particles is also shown.

anti-glutamate antibody, used in this study, labelled GABA-IR processes. A paired t-test (Table 4.4) was used to assess if the density of gold beads per  $\mu\text{m}^2$  area of identified GABA-IR processes in a section labelled with anti-GABA antibody was significantly different from the density of gold beads per  $\mu\text{m}^2$  area of the same process in a serial section labelled with anti-glutamate antibody.

## **4.3. RESULTS**

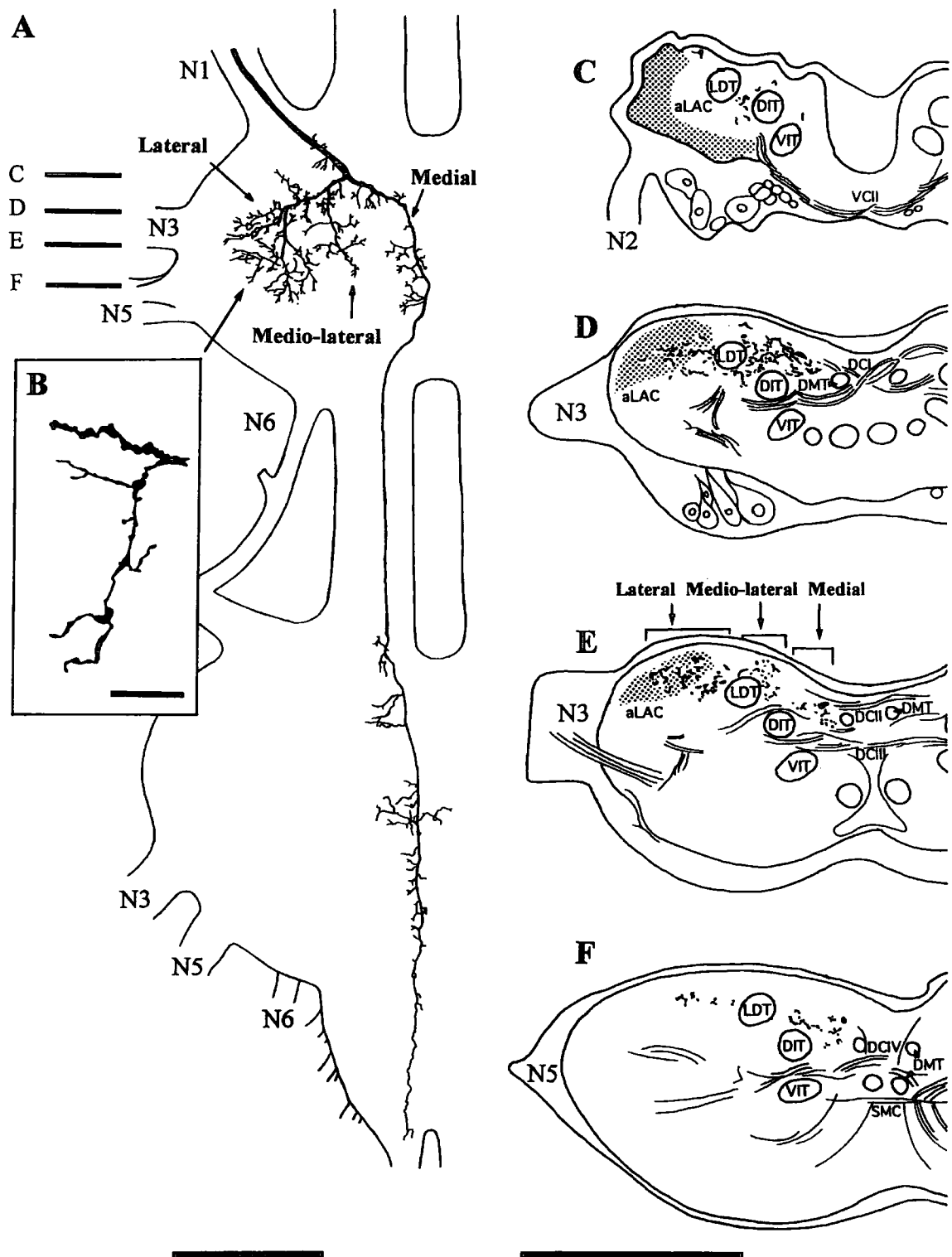
### **4.3.1. Morphology of the fSR**

To provide a background for the analysis of the distribution of GABA-IR inputs to the fSR terminals, a brief description of the central branching pattern of the fSR in the mesothoracic ganglion of the adult *Locusta migratoria* is given. The morphology of the fSR was revealed by back-filling the fSR with cobalt chloride and examining both wholemounts of the thoracic ganglia and transverse serial sections through the mesothoracic ganglion, using a binocular light microscope fitted with a camera lucida attachment and drawing tube.

Each fSR neuron sends a large axon (approximately 10  $\mu\text{m}$  in diameter) from the periphery towards the CNS in branch N1D<sub>2</sub> (Campbell, 1961). Before the fSR axon enters the CNS it bifurcates, sending one branch into the prothoracic ganglion via prothoracic N6 and one branch into the mesothoracic ganglion via N1 (Figure 4.1A). No other sensory afferent from the wing bifurcates in this way into prothoracic N6.

**Figure 4.1. Morphology of the fSR.** **A:** Camera lucida drawing of the fSR taken from a wholemount preparation showing the position of the fSR within the mesothoracic and metathoracic ganglia. Anterior is to the top. After entering the mesothoracic ganglion via nerve 1 (N1), the fSR divides into a medial branch and a lateral branch. The medial branch runs close to the dorsal midline, into the meso-metathoracic connective, and then into the metathoracic ganglion. The lateral branch bifurcates into a medio-lateral branch and lateral branch. The medio-lateral branch terminates just anterior to the level of emergence of nerve 5 (N5). The extensive lateral branch arborizations extend posteriorly to the emergence of N5. The levels within the ganglia at which transverse sections shown in C-F and ultrathin sections for electron microscopy were taken are indicated by the thick horizontal lines. **B:** High magnification of the fine terminal branch indicated by the arrow in A. Note the characteristic swellings along its length. C-F: Camera lucida drawings taken from selected transverse sections through a single mesothoracic ganglion in which the fSR has been back-filled with cobalt chloride. Each drawing is a composite of serial 5  $\mu$ m sections taken at each level. The anterior lateral association centre (aLAC) is represented by the stippled area. Dorsal is to the top. **C:** The fSR has just entered the mesothoracic ganglion, and its main branch is towards the dorsal edge of the ganglion. **D:** The fSR has subdivided into medial, medio-lateral, and lateral branches. The lateral branches can be seen in aLAC. It is difficult to distinguish between the main arborizations of each main branch. **E:** The positions of the medial, medio-lateral, and lateral branches are indicated. The medio-lateral branches lie dorsal to the dorsal intermediate tract (DIT) and medial to the lateral dorsal tract (LDT). **F:** The medial and lateral branches can be seen clearly, but the medio-lateral branches have not extended this far posteriorly. The nomenclature of the commissures, tracts, and neuropils is that used by Tyrer and Gregory (1982) and Pflüger et al. (1988). Abbreviations are shown in Table 4.1. Scale bars = 200  $\mu$ m in A, C-F, 20  $\mu$ m in B.

**Figure 4.1. Morphology of the fSR.**



The fSR branch in N1 enters the mesothoracic ganglion dorsally (Figures 4.1A,C). After entering the ganglion it projects medially towards the midline before bifurcating into a large medial branch and a smaller lateral branch (Figure 4.1A). The large medial branch continues projecting medially to approximately the level of the emergence of nerve 3 (N3), before turning posteriorly and running parallel and close to the dorsal midline (Figures 4.1A,D-F). The medial branch continues projecting posteriorly through the mesothoracic ganglion and into the ipsilateral meso-metathoracic connective (Figure 4.1A). It enters the metathoracic ganglion and continues to project posteriorly close to the midline before terminating in the fused first three abdominal neuromeres (Figure 4.1A). The medial branch, gives rise to numerous short fine laterally and ventrally directed processes along its length, in the meso- and metathoracic ganglia (Figure 4.1A).

The lateral branch of the fSR projects laterally and posteriorly for a short distance before bifurcating into a medio-lateral branch and a lateral branch (in some preparations the medio-lateral branch arises directly from the main axon and not as a side branch of the lateral branch). The medio-lateral branch projects posteriorly between the main medial and lateral branches giving rise to several short branches processes (Figure 4.1A). These short processes extend into the neuropil lying dorsal to the dorsal intermediate tract (DIT) and medial to the lateral dorsal tract (LDT) (Figures 4.1D,E). The field of medio-lateral branches begins anterior to the level of emergence of N3 and terminates anterior to the level of the emergence of nerve 5 (N5) (Figure 4.1A).



The lateral branch of the fSR repeatedly subdivides to form elaborate arborizations in the dorsal lateral neuropil of the mesothoracic ganglion (Figures 4.1A,D-F). The lateral field of branches extends laterally from LDT to the lateral edge of the dorsal neuropil throughout the anterior lateral association centre (aLAC) (Figures 4.1A,D-F). The lateral branches terminate at the level of the emergence of N5 (Figures 4.1A,F).

The fine branches of the fSR have a characteristic beaded appearance (Figure 4.1B) as a result of swellings along their length and at their terminals. These swellings are the sites of vesicle accumulation and output synapses (see below).

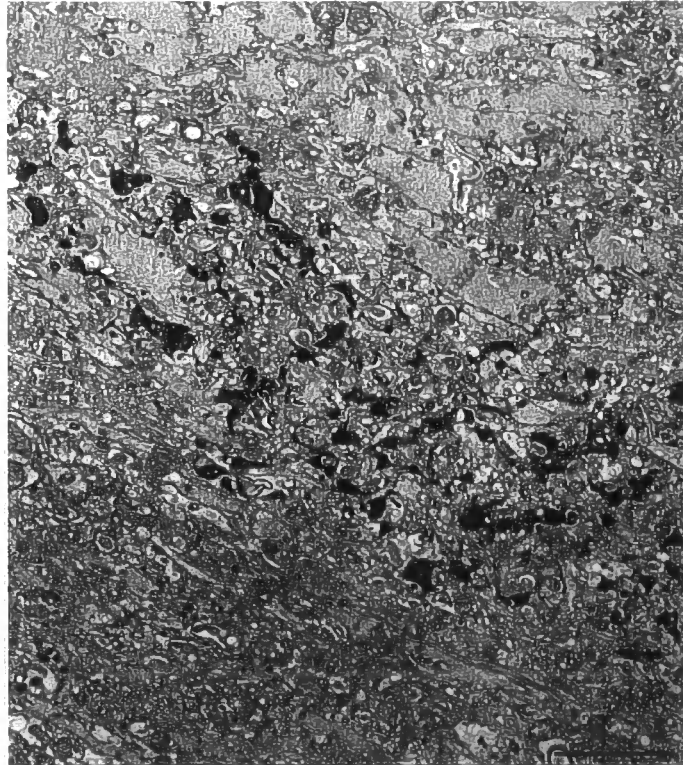
#### **4.3.2. Distribution of synapses on the fSR**

The distribution of synapses involving the fSR were examined by analysing the number and type of synaptic contacts (inputs or outputs, GABA-IR or non-GABA-IR) on the medial, medio-lateral and lateral fields of branches of the fSR (Figures 4.2.-4.5) at each of the levels indicated in Figure 4.1A,C,E,F (synaptic interactions were not analysed at level D as the three main fields of branches could not be clearly discriminated from each other at this level).

The fSR was back-filled with HRP so the fSR could be clearly identified within the ultra-thin transverse sections taken from mesothoracic ganglia by the presence of the electron-dense HRP-DAB reaction product (Figure 4.2). The fSR profiles ranged in size from 0.3  $\mu\text{m}$  to 3  $\mu\text{m}$ . The larger profiles represent sections through the bead-like axonal swellings seen in the cobalt back-filled wholemounts (Figure 4.1A,B) that

**Figure 4.2. HRP-filled fSR profiles in the locust mesothoracic ganglion.** Electron micrograph of a transverse section through the mesothoracic ganglion at level E. The profiles of the fSR medio-lateral branch are uniquely identifiable by the presence of electron-dense HRP-DAB reaction product. Dorsal is to the top. Lateral is to the left. Scale bar = 5  $\mu\text{m}$ .

**Figure 4.2. HRP-filled fSR terminals in the locust mesothoracic ganglion.**



are sites of multiple input and output synapses (Figure 4.4A,C). Synaptic contacts involving the fSR were identified by the following established criteria: the presence of a presynaptic density or bar, presynaptic vesicles, and postsynaptic membrane thickening.

One hundred and eleven synapses involving the fSR were identified in a single specimen (Table 4.5). Of these, 69 (62%) were inputs to the fSR and 42 (38%) were outputs from the fSR, representing a ratio of approximately three inputs to two outputs. Twenty three (20.5%) of all synapses found were located on the side branches of the fSR medial branch, 26 (23.5%) were on the medio-lateral branches and 62 (56%) were on the lateral branches. Inputs and outputs were located on medial, medio-lateral and lateral branches of the fSR in the following ratios: on side branches of the medial branch, the ratio of inputs to outputs was 2:1; on the medio-lateral branches, the ratio of inputs to outputs was 3:2; and on the lateral branches, the ratio of inputs to outputs was 3:2.

The distribution of synapses on the lateral field of branches was similar at all the levels examined (Figure 4.1C,E,F) throughout the region of dorso-lateral neuropil, where the lateral branch extends its arbors (i.e. from the lateral edge of the dorsal neuropil throughout aLAC and posteriorly to the level of N5). The distribution of input and output synapses on medio-lateral branches anterior to the level of emergence of N5 (medio-lateral branches do not extend beyond this level) was also similar at all levels examined. No input or output synapses were made directly onto the main medial fSR branch, but were restricted to side branches of the main medial branch. The synapses on the medial side branches were concentrated on branches

**Table 4.5. Number and type of synaptic contacts onto and from the fSR at its main field of branches analysed in sections labelled with anti-GABA antibody from a single specimen.**

Synapse type	Location			Total
	<i>medial</i>	<i>medio-lateral</i>	<i>lateral</i>	
Inputs to the fSR				
GABA-immunoreactive inputs	8	5	17	30
Non-immunoreactive inputs	8	11	20	39
Total inputs	16	16	37	69
Outputs from the fSR				
GABA-immunoreactive	2	0	1	3
Non-immunoreactive	5	10	24	39
Total outputs	7	10	25	42
Grand total	23	26	62	111

anterior to the level of emergence of N5; only one output was found at level F (Figure 4.1A, F).

There was no evidence of spatial segregation of inputs and outputs on particular branches of the fSR, although there was a higher proportion of synapses located on the lateral branches of the fSR at all levels examined. This indicates that a greater proportion of the synaptic interactions involving the fSR occur in the dorso-lateral neuropil and throughout aLAC.

#### **4.3.3. GABA-IR inputs to the fSR**

Transverse sections through the mesothoracic ganglion, labelled with anti-GABA antibody, showed numerous immunolabelled processes, indicated by a high density of gold beads making synaptic inputs onto HRP-filled processes of the fSR (Figures 4.3,4.4). The mean density of gold beads over GABA-IR processes was significantly greater ( $p < 0.0001$ ) than the background levels of gold beads (see Table 4.2). The distribution of GABA-IR and non-GABA-IR inputs onto medial, medio-lateral, and lateral branches of the fSR was analysed at each of the levels indicated in Figure 4.1A,C,E,F.

Thirty of the 69 inputs to the fSR (43%) were from GABA-IR processes. The majority of these, 57% (17 out of 30) were located on lateral branches of the fSR. Of the remainder, 16% (5 out of 30) were made onto medio-lateral branches, and 27% (8 out of 30) were made onto side branches of the main medial branch. No inputs, however, were made directly onto the main medial fSR branch. GABA-IR inputs

onto the lateral branches occurred at all levels examined but the GABA-inputs to the medial and medio-lateral branches were restricted to branches anterior to the level of the emergence of N5.

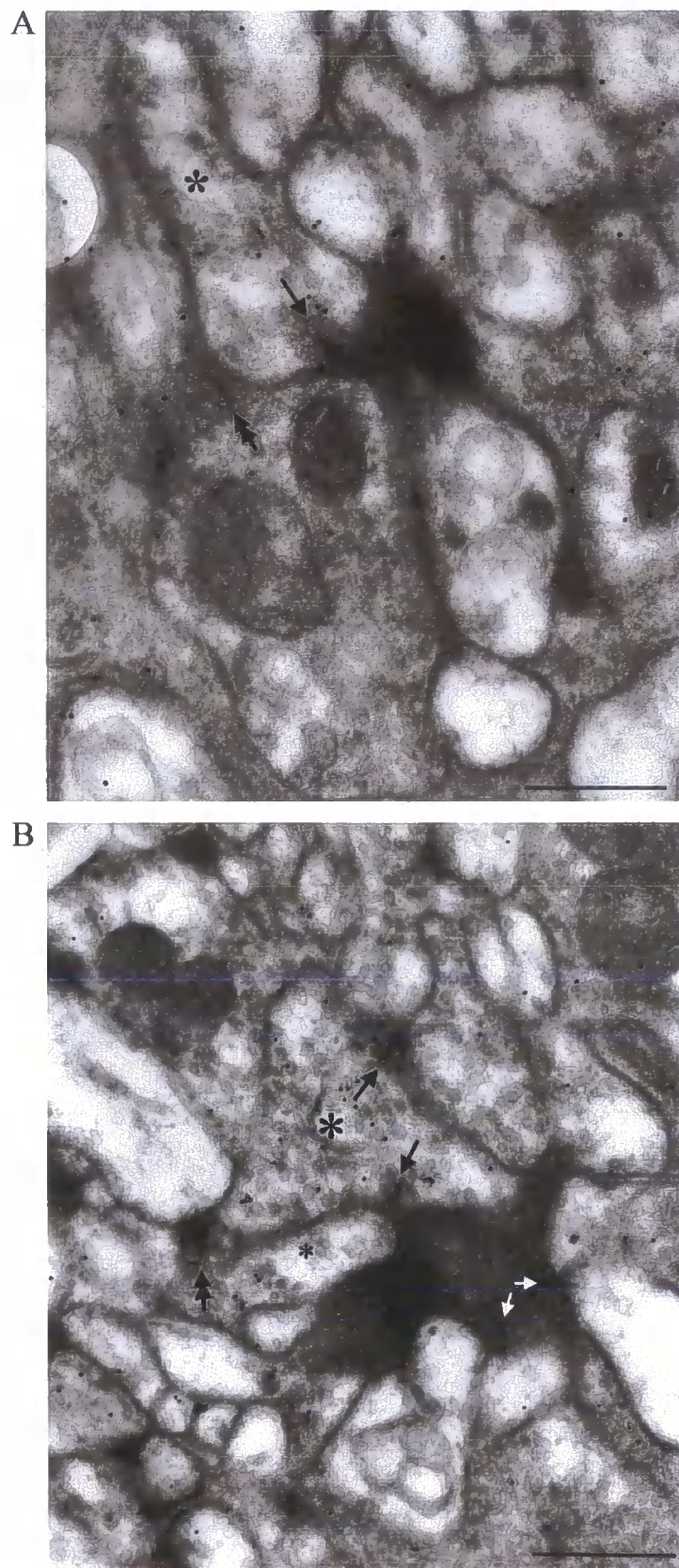
The GABA-IR processes that made synaptic contact with the fSR ranged in size from small terminal neurites (0.3  $\mu\text{m}$ ) to large processes (2-4  $\mu\text{m}$  in diameter). The GABA-IR processes contained numerous small (30-50 nm), agranular pleomorphic vesicles (Figures 4.3, 4.4). Some also contained, in addition to the small vesicles, a few larger (150 nm) dense-cored vesicles. The fSR profiles which received synaptic inputs from the GABA-IR processes were non-GABA-IR and contained small (30-50 nm) spherical vesicles (Figure 4.5B).

The majority of synaptic interactions between GABA-IR processes and fSR profiles involved postsynaptic dyads (Figures 4.3, 4.4). At some dyads, the GABA-IR process was presynaptic to the fSR and a non-GABA-IR process. For example, in Figure 4.3A, the GABA-IR process (asterisk) is presynaptic to a lateral branch of the fSR and a non-GABA-IR process. The non-GABA-IR process in turn makes a reciprocal synapse (double arrow) back onto the GABA-IR process (asterisk). At other dyadic contacts, the second member of the postsynaptic dyad was also GABA-IR. For example, in Figure 4.3B, the GABA-IR process (large asterisk) is presynaptic to a lateral branch of the fSR and a second GABA-IR process (small asterisk). The latter GABA-IR process (small asterisk) makes a reciprocal synapse (double arrow) back onto the former GABA-IR process (large asterisk). Frequently, synaptic inputs from GABA-IR processes to the fSR were in close proximity to outputs from the fSR, indicating neurotransmitter release from the fSR terminal is

**Figure 4.3. GABA-immunoreactive processes make synaptic inputs onto the fSR.** Electron micrographs of transverse sections through the mesothoracic ganglion at level F showing profiles of the fSR lateral branch filled with horseradish peroxidase. The electron dense profiles receive synaptic inputs from GABA-immunoreactive (GABA-IR) processes, indicated by a high density of gold beads. **A:** A GABA-IR process (asterisk) making a synaptic input (single arrow) onto a small fSR process and a non-GABA-IR profile. The non-GABA-IR profile makes a synapse (double arrow) back onto the GABA-IR process (asterisk). **B:** A GABA-IR process (large asterisk) making synaptic inputs (single black arrows) onto a fSR process and a number of GABA-IR processes. One of the postsynaptic GABA-IR processes (small asterisk) makes a reciprocal synapse (double black arrow) back onto the former GABA-IR process (large asterisk). The fSR process makes synaptic outputs (white arrows) onto non-GABA-IR profiles. Scale bar = 0.5  $\mu\text{m}$ .

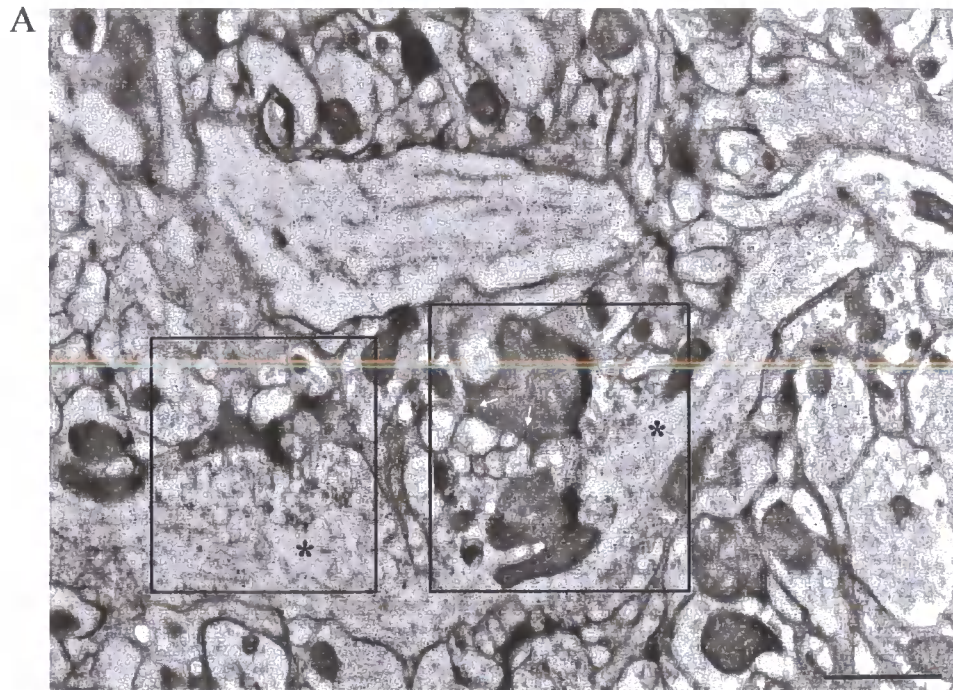


**Figure 4.3. GABA-immunoreactive processes make synaptic inputs onto the fSR.**



**Figure 4.4. A single GABA-immunoreactive process makes synaptic inputs onto multiple fSR profiles.** Electron micrographs of a transverse section through the mesothoracic ganglion at level F showing synaptic contacts between a large GABA-IR process and lateral branches of the fSR. **A:** Low magnification of a large GABA-IR neuron (asterisks) receiving synaptic outputs (white arrows) from the fSR. Scale bar = 1  $\mu\text{m}$ .

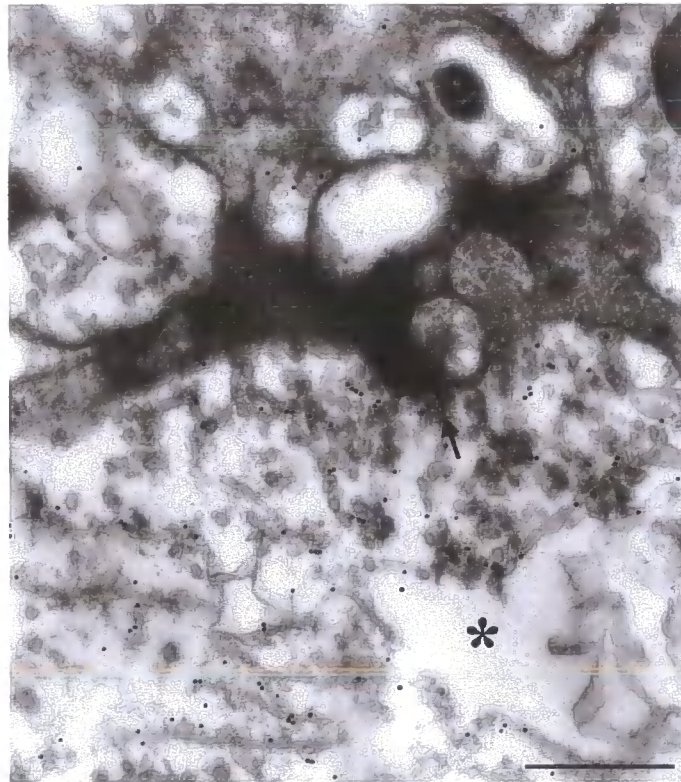
**Figure 4.4. A single GABA-immunoreactive process makes synaptic inputs onto multiple fSR profiles.**



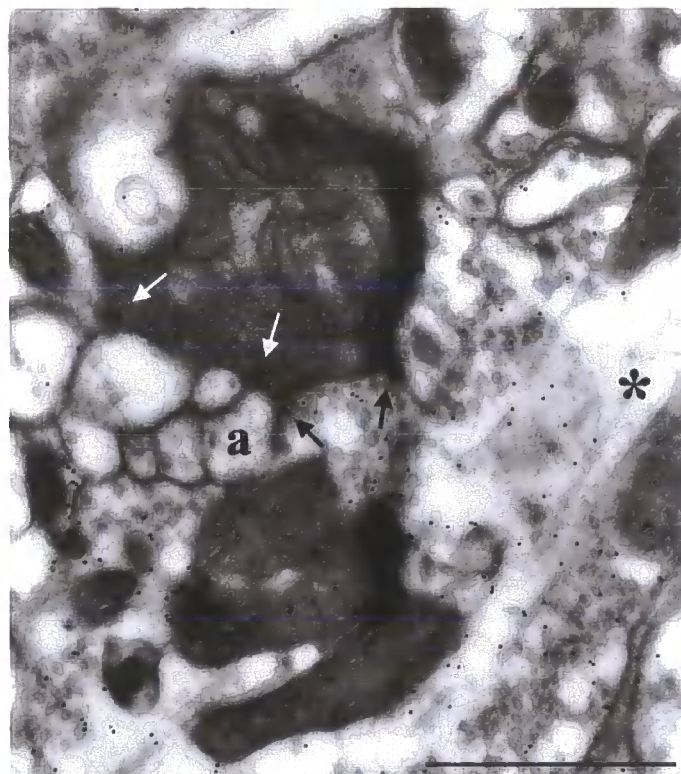
**B:** High magnification of the boxed area on the left in A. The large GABA-IR neuron (asterisk) makes an input synapse (arrow) onto a small fSR profile. **C:** High magnification of the boxed area on the right in A. The GABA-IR process (asterisk) makes synaptic inputs (black arrows) onto a fSR process and non-GABA-IR profiles. The fSR process makes synaptic outputs (white arrows) onto non-GABA-IR profiles. One of the non-GABA-IR profiles (a) also receives a synaptic input from the GABA-IR process (asterisk). Scale bars = 0.5  $\mu\text{m}$ .



**B**



**C**



tightly controlled by the GABA-IR processes. In Figure 4.4C, for example, the large GABA-IR process (asterisk) makes multiple synaptic inputs (black arrows) onto a HRP-filled fSR process. The fSR process in turn makes synaptic outputs (white arrows) onto non-GABA-IR processes, one of which (a) also receives a synaptic input from the same large GABA-IR process (asterisk). At such sites of multiple input and output synapses, the fSR profiles were frequently enlarged to accommodate accumulations of vesicles. These swellings were responsible for the characteristic beaded appearance of terminal branches of the fSR evident in cobalt back-filled preparations (Figure 4.1B).

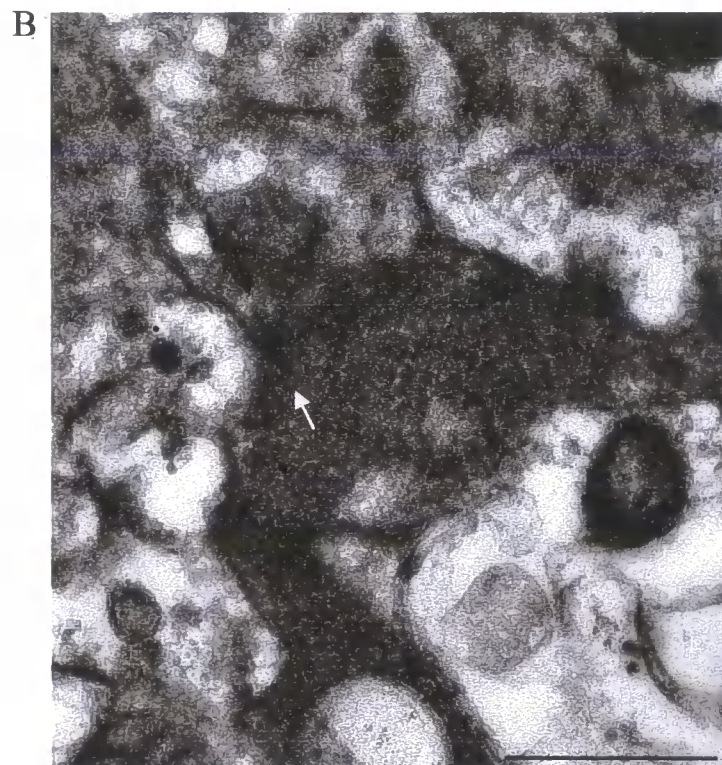
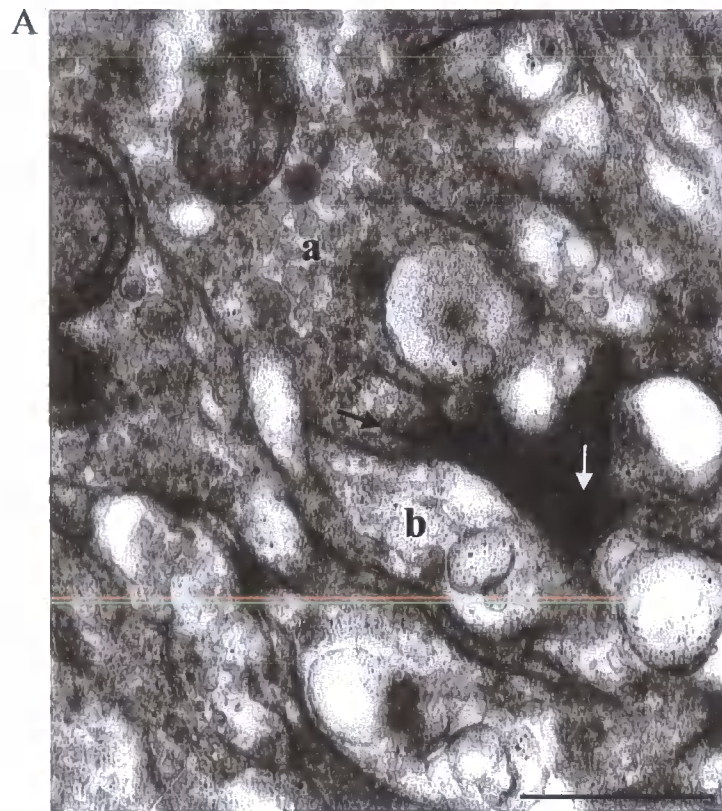
#### **4.3.4. Non-GABA-IR inputs onto the fSR**

Of the all the synaptic inputs to the fSR, 57 % (39 out of 69) were from non-GABA-IR (Figure 4.5). Of these, 51 % (20 out of 39) were located on lateral branches of the fSR, 28 % (11 out of 39) were on medio-lateral branches, and 21 % (8 out of 39) were located on the side branches of the medial branch. No inputs, however, were made onto the main medial branch.

The non-GABA-IR processes presynaptic to the fSR could be divided into two groups on the basis of their vesicle content. Some of the non-GABA-IR processes contained predominantly clear spherical vesicles (40-60 nm) (Figure 4.5A) whereas others contained, in addition to clear vesicles, numerous dense-cored vesicles measuring 80-100 nm (Figure 4.5C). In all cases in which a non-GABA-IR process was presynaptic to an fSR profile, the second member of the postsynaptic dyad was also not immunoreactive for GABA (Figure 4.5). In Figure 4.5A, for example, the non-

**Figure 4.5. Non-immunoreactive processes make synaptic inputs onto the fSR.** Electron micrographs of transverse sections through the mesothoracic ganglion at level E. **A:** A non-GABA-IR process (a) containing predominantly clear spherical vesicles, makes a synaptic input (black arrow) onto a fSR terminal of the medio-lateral branch. The fSR terminal makes an output synapse (white arrow) onto a non-GABA-IR process (b), which also receives a synaptic input (black arrow) from the former non-GABA-IR process (a). **B:** High magnification of a fSR process from the lateral branch containing spherical vesicles. The fSR process makes an output synapse (white arrow) onto non-GABA-IR processes. One of the non-GABA-IR processes makes a reciprocal synapse (black arrow) back onto the fSR process. Scale bars = 0.5  $\mu\text{m}$ .

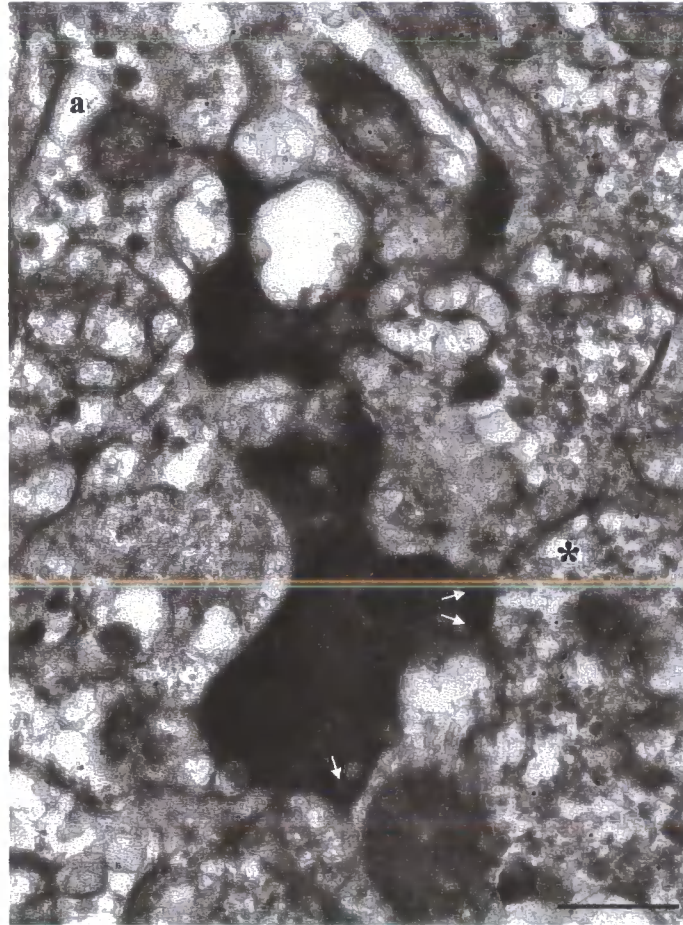
**Figure 4.5. Non-immunoreactive processes make synaptic inputs onto the fSR.**





**C:** A HRP-filled medial branch of the fSR receives an input (black arrow) from a non-GABA-IR process (a) containing a number of spherical dense-cored vesicles. The fSR profile also makes synaptic outputs (white arrows) onto other non-GABA-IR processes and a large GABA-IR process (asterisk). Scale bars = 0.5  $\mu\text{m}$ .

C



GABA-IR process (a) is presynaptic to a medio-lateral terminal of the fSR and another non-GABA-IR process (b), which also receives an output (white arrow) from the fSR terminal. No cases in which a non-GABA-IR process was presynaptic to an fSR profile and a GABA-IR process were found.

#### **4.3.5. Synaptic outputs from the fSR**

Of the 111 synapses identified on the fSR, 38% (42 out of 111) were outputs to other neurons. Of these outputs, 60% (25 out of 42) were located on lateral branches of the fSR, 24% (10 out of 42) were on medio-lateral branches, and 16% (7 out of 42) on were on the side branches of the main medial branch.

Most of the outputs from the fSR (93%) involved postsynaptic dyads in which both postsynaptic members were non-GABA-IR. For example, in Figure 4.5B, a lateral branch terminal of the fSR makes an output synapse (white arrow) onto two non-GABA-IR processes. One of these non-GABA-IR processes makes a reciprocal synapse (black arrow) back onto the fSR terminal. Only 7% of outputs from the fSR were to GABA-IR processes. In Figure 4.5C, for example, a medial branch terminal of the fSR makes synapses (white arrows) onto a large GABA-IR process (asterisk) and onto non-GABA-IR processes.

#### **4.3.6. Glutamate-IR synaptic inputs to the fSR**

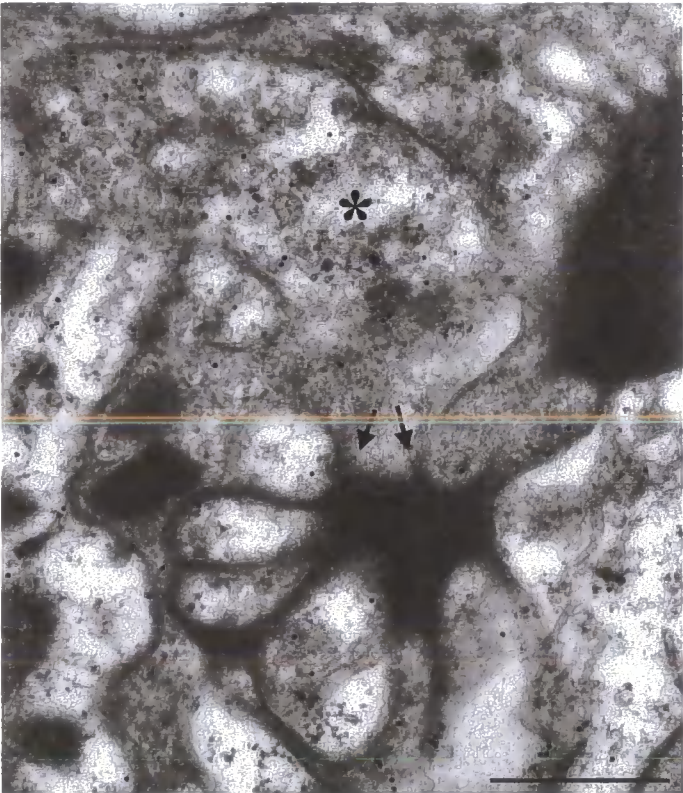
The ICC study described above reveals that the fSR receives synaptic inputs from GABA-IR, which suggests that the fSR may be presynaptically modulated by GABA.

However, 57% of the synaptic inputs to the fSR are from processes that are not immunoreactive for GABA, indicating that the fSR may also be modulated by other neurotransmitters. A number of other insect sensory afferents have also been reported to receive synaptic inputs from non-GABA-IR processes (locust cercal afferents, Watson, 1990; locust hair plate afferents, Watson et al., 1991; locust campaniform sensilla afferents, Watson and England, 1991; locust FeCO afferents, Watson et al., 1993; locust prosternal filiform afferents, Watson and Pflüger, 1994; bushcricket and cricket auditory organ afferents, Hardt and Watson, 1999) and some of these were found to be immunoreactive for glutamate (Watson et al., 1991; Watson and Pflüger, 1994; Hardt and Watson, 1999). The vesicles in glutamate-IR processes identified in these and other EM ICC studies (Watson, 1988; Watson et al., 1991; Watson and Pflüger, 1994; Hardt and Watson, 1999) are spherical and agranular, making them morphologically similar to those in many of the non-GABA-IR processes presynaptic to the fSR (Figure 4.5A). To determine if any of the non-GABA-IR processes presynaptic to the fSR are glutamate-IR an additional ICC study was performed.

Transverse sections taken through mesothoracic ganglia containing HRP-filled fSR, which were labelled with anti-glutamate antibody, showed numerous immunolabelled processes indicated by a concentrated covering of gold beads. The density of gold beads over glutamate-IR processes was significantly greater ( $p < 0.0001$ ) than the background levels of gold beads (see Table 4.3). Numerous glutamate-IR processes made synaptic inputs onto the HRP-filled profiles of the fSR (Figure 4.6). The profiles that were glutamate-IR and made synaptic inputs to the fSR ranged in size from 0.3  $\mu\text{m}$  to 3.5  $\mu\text{m}$ . The glutamate-IR processes that made synaptic inputs to the fSR contained predominantly round agranular vesicles measuring 40-70 nm (Figure 4.6),

**Figure 4.6. The fSR receives synaptic inputs from a glutamate-immunoreactive process.** Electron micrograph of a transverse section through the mesothoracic ganglion at level E showing a HRP-filled profile of the fSR lateral branch. The electron-dense fSR profile receives synaptic inputs (black arrows) from a glutamate-IR neuron (asterisk), indicated by a high density of gold beads. The glutamate-IR process (asterisk) contains spherical agranular vesicles. Scale bar = 0.5  $\mu\text{m}$ .

**Figure 4.6. The fSR receives synaptic inputs from a glutamate-immunoreactive process.**



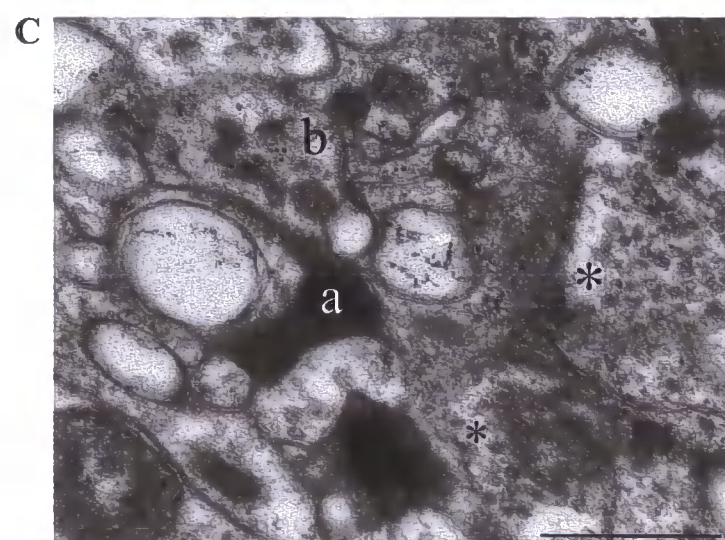
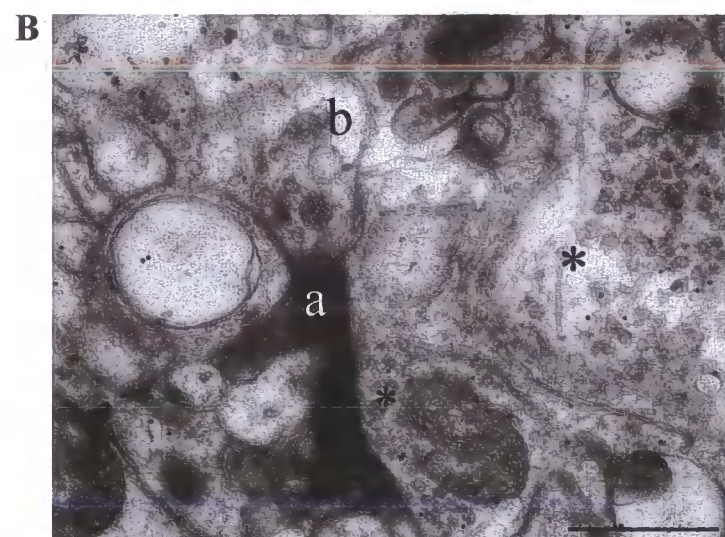
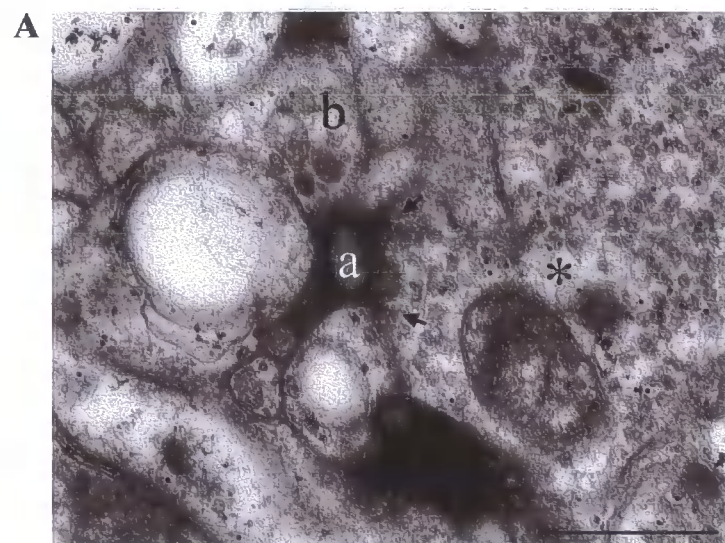
although some also contained a small number of large granular vesicles measuring 60-140 nm. Many of the synaptic inputs from glutamate-IR processes to the fSR terminals were dyadic and involved a second postsynaptic member opposite the presynaptic bar. The majority of the second members of the dyad were non-glutamate-IR. For example, in Figure 4.6., the glutamate-IR process (asterisk) makes synaptic inputs (arrows) onto an electron-dense profile of the fSR lateral branch and non-glutamate-IR processes.

Anti-glutamate antibodies have been reported also to label GABA-IR processes (Watson et al., 1991). Therefore, the selectivity of the anti-glutamate antibody was tested on serial sections stained alternatively with anti-glutamate and anti-GABA antibody to check that GABA-IR profiles were not also glutamate-IR and vice versa. The density of gold beads over identified GABA-IR processes in serial sections incubated alternatively with anti-glutamate and anti-GABA antibodies were examined (see Table 4.4). Identified GABA-IR processes (covered in a high density of gold beads) in sections incubated with anti-GABA antibody were only covered in background levels of gold particles in sections incubated with anti-glutamate antibody (see Table 4.4). For example, in Figure 4.7, processes that are GABA-IR (asterisks) in sections incubated with anti-GABA antibody (Figure 4.7A,B) are not immunoreactive in sections incubated with anti-glutamate antibody (Figure 4.7C). Likewise, a glutamate-IR profile (b) that is in close contact with an fSR process (Figure 4.7C) is not immunoreactive in sections incubated with anti-GABA antibody (Figure 4.7A,B). These results demonstrate that the anti-glutamate antibody used in this study does not label GABA-IR processes.

**Figure 4.7. GABA-immunoreactive processes are not immunoreactive for glutamate.** Electron micrographs of semi-serial transverse sections through the mesothoracic ganglion at level E showing the same fSR profiles and adjacent neurons. **A:** Electron micrograph of a section labelled with anti-GABA antibody. A large process (asterisk) is immunoreactive for GABA, indicated by a high density of gold beads. The GABA-IR process makes synaptic inputs (arrows) onto a small fSR profile (a) and non-GABA-IR processes. **B:** Electron micrograph of a semi-serial section also labelled with anti-GABA antibody. In this section the GABA-IR process (asterisk) shown in **A** has branched and is shown as a small GABA-IR profile (small asterisk) adjacent to a large GABA-IR profile (large asterisk). **C:** Electron micrograph of a serial section labelled with anti-glutamate antibody. Neither the large or small GABA-IR profiles (asterisks) are immunoreactive for glutamate. The fSR process (a) is in close contact with a glutamate-IR process (b) containing large dense-cored vesicles. Comparison with **A** and **B** shows that the glutamate-IR process (b) is not immunoreactive for GABA. Scale bars = 0.5  $\mu\text{m}$ .



**Figure 4.7. GABA-immunoreactive processes are not immunoreactive for glutamate.**



#### **4.3.7. Non-glutamate-IR synaptic inputs to the fSR**

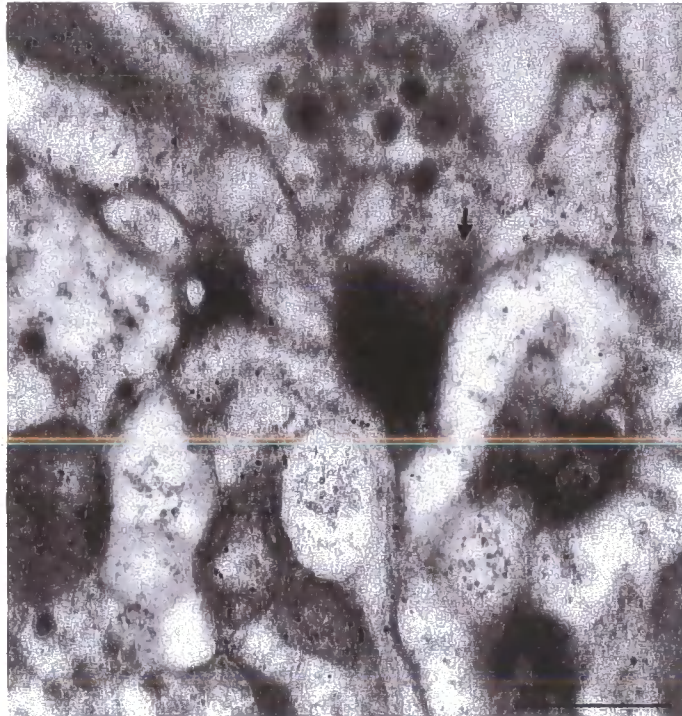
Examination of the transverse serial sections labelled with either anti-glutamate or anti-GABA antibody revealed that the fSR also receives inputs from processes that are immunoreactive neither for glutamate nor GABA (Figure 4.8). The non-glutamate-IR processes that made synaptic inputs to the fSR terminals ranged in diameter from 0.1  $\mu\text{m}$  to 1.5  $\mu\text{m}$ . Some of the non-glutamate-IR processes that made synaptic inputs to the fSR profiles contained small agranular vesicles whereas, others contained large dense-cored vesicles (Figure 4.8). The majority of the synaptic inputs from non-glutamate-IR processes to the fSR profiles were also dyadic. In Figure 4.8, for example, a non-glutamate-IR process containing large dense-cored vesicles makes a synaptic input (arrow) onto a fSR profile of the lateral branch and another non-glutamate-IR process.

#### **4.3.8. Proportions of synaptic inputs to the fSR**

A full analysis of the distribution of synaptic contacts involving the fSR, at each of the transverse levels described above in the GABA-ICC study (4.3.2.-4.3.5.), was not conducted for the glutamate ICC study. Instead, the number of inputs from glutamate-IR and non-glutamate-IR inputs onto medial, medio-lateral, and lateral branches of the fSR at level E was analysed in a single specimen (Table 4.6). The synaptic contacts involving the fSR were examined at this level because the medial, medio-lateral and lateral branches of the fSR are all present and clearly identifiable as separate fields at this level.

**Figure 4.8. The fSR receives a synaptic input from a non-glutamate-immunoreactive process.** Electron micrograph of a transverse section through the mesothoracic ganglion at level E showing a HRP-filled fSR profile of the lateral branch. The fSR electron dense process receives a synaptic input (black arrow) from a non-glutamate-IR process containing a number of large dense-cored vesicles. Scale bar = 0.5  $\mu\text{m}$ .

**Figure 4.8. The fSR receives a synaptic input from a non-glutamate-immunoreactive process.**



**Table 4.6. The number and type of synaptic inputs onto the medial, medio-lateral and lateral branches of the fSR at level E, analysed in sections labelled with anti-glutamate antibody from a single specimen.**

Synapse type	Location			Total
	Medial	Medio-lateral	Lateral	
Inputs to the fSR				
Glutamate-immunoreactive inputs	3	3	3	9
Non-immunoreactive inputs	2	7	5	14
Total inputs	5	10	8	23

Of the 23 synaptic inputs to the fSR 39 % (9 out of 23) were from neurons that were glutamate-IR and 61 % (14 out of 23) were from neurons that were non-glutamate-IR. Equal numbers of the glutamate-IR inputs were located on the medial, medio-lateral and lateral branches. Of the 14 non-glutamate-IR inputs to the fSR, 14 % were located on the side branches of the main medial branch, 50% were located on the medio-lateral branches and 36% on the lateral branches. There was no evidence of spatial segregation between the different types of inputs.

#### **4.4. DISCUSSION**

##### **4.4.1. Presynaptic inputs to the fSR**

The results presented in this chapter show that the fSR of *Locusta migratoria* receives synaptic inputs from processes that are clearly and specifically immunolabelled with anti-GABA antibody (Figures 4.3-4.4). The GABA-IR processes presynaptic to the fSR terminals contain predominantly small, pleomorphic agranular vesicles, which are characteristic of GABA-IR processes examined in other EM ICC studies in the locust (Watson, 1988; Watson, 1990; Watson et al., 1991, 1993; Watson and Pflüger, 1994; Pflüger and Watson, 1995; Leitch and Laurent, 1996), cockroach (Distler and Boeckh, 1997), bushcricket (Hardt and Watson, 1999), cricket (Watson and Hardt, 1996; Hardt and Watson, 1999) and earthworm (Telkes et al., 1996). Some also possessed a few larger dense-cored vesicles that are also characteristic of GABA-IR processes in the locust (Watson, 1988; Watson et al., 1991, 1993; Watson and Pflüger, 1994; Pflüger and Watson, 1995; Leitch and Laurent, 1996) and cockroach (Distler and Boeckh, 1997).

Analysis of the relative number and distribution of both GABA-IR and non-GABA-IR processes presynaptic to the fSR has shown that the majority of the identified inputs (37 of 69, or 54%) are located on the lateral field of branches of the fSR. This indicates that a greater proportion of the presynaptic inputs to the fSR occur in the dorso-lateral neuropil and throughout aLAC. There is, however, no evidence that different types of synaptic input are represented exclusively in any particular region of the arborization; GABA-IR and non-GABA-IR inputs are intermingled at each of the levels examined.

The relative density of GABA-IR inputs to the fSR (43%) is markedly less than the proportion of GABA-IR inputs reported at other insect sensory afferents. For example, 93% of inputs to locust hair plate afferents (Watson et al., 1991), 72% of inputs to campaniform sensilla afferents (Watson and England, 1991), 78% of inputs to femoral chordotonal organ afferents (Watson et al., 1993), 51% of inputs to prosternal filiform afferents (Watson and Pflüger, 1994) and 65% of inputs to bushcricket auditory afferents (Hardt and Watson, 1999) are from GABA-IR processes. The significance of the higher proportion of presynaptic GABA-IR inputs to these former types of sensory afferents is unclear at present but may indicate that presynaptic inhibition by GABA plays a relatively more important role in the modulation of these afferent outputs.

In the fSR of *Locusta migratoria*, a higher proportion (57%) of inputs are non-GABA-IR. The effectiveness of synaptic transmission from the fSR afferent could thus be potentially modulated by inputs from these non-GABA-IR neurons. A further ICC study revealed that some of the non-GABA-IR inputs presynaptic to the fSR were

immunoreactive for glutamate (Figure 4.6). Synaptic inputs to the fSR from glutamate-IR processes were intermingled with inputs from non-glutamate-IR processes on the lateral, medio-lateral and side branches of the main medial branch of the fSR at level E. The percentage of glutamate-IR inputs to the fSR (39%) is similar to the percentage of glutamate-IR inputs to locust prosternal filiform afferents (40%) (Watson and Pflüger, 1994). Other types of insect sensory afferents, however, receive a lower percentage of synaptic inputs from glutamate-IR processes (Watson et al., 1991; Hardt and Watson, 1999). Twenty-five percent of synaptic inputs to cricket auditory afferents are glutamate-IR (Hardt and Watson, 1999) and only one synaptic input to the locust hair plate afferents was found to be glutamate-IR (Watson et al., 1991). Like the fSR, the locust prosternal filiform afferents also receive a similar percentage inputs from glutamate-IR and GABA-IR (Watson and Pflüger, 1994) (the fSR receives 43% GABA-IR inputs and 39% glutamate-IR inputs, the prosternal filiform afferents receive 51% GABA-IR and 40% glutamate-IR inputs). This indicates that glutamate and GABA may have equally important roles in modulating the fSR and prosternal filiform afferents.

GABA-IR inputs account for 43% of the inputs to the fSR and glutamate-IR account for 39%. This means a proportion of inputs to the fSR (approximately 18%) are not immunoreactive for GABA or glutamate, indicating other neurotransmitters may be involved in presynaptically modulating the fSR. Similar findings have been reported for other insect afferents; approximately 10-15% of the inputs to cricket auditory afferents (Hardt and Watson, 1999), 9% of the inputs to locust prosternal filiform afferents (Watson and Pflüger, 1994) and 7% of the inputs to locust hair plate afferents (Watson et al., 1991) are not labelled by either glutamate or GABA antibodies. The processes



presynaptic to the fSR that were not immunoreactive for glutamate or GABA contained either small agranular vesicles or in addition to the small agranular vesicles some larger dense-cored vesicles (Figure 4.8). Processes presynaptic to the locust prosternal filiform afferents that are not immunoreactive for GABA or glutamate have also been reported to contain large granular vesicles (Watson and Pflüger, 1994). The identity of the neurotransmitter in this type of process is unknown but neurons which contain the biogenic amines, 5HT (Nässel and Elekes, 1984; Nässel et al., 1985), dopamine (Elekes et al., 1987) and octopamine (Watson, 1984; Lee and Wyse, 1991) contain predominantly large granular vesicles. This suggests those processes presynaptic to the fSR and prosternal filiform afferents which are not GABA- or glutamate-IR may contain biogenic amines. Caution must be used though in trying to identify the neurotransmitter in a process from the morphology of its vesicles as neurotransmitters may be associated with more than one vesicle type. Immunocytochemistry has to be used therefore, to identify the neurotransmitter content of a neuron.

#### **4.4.2. Neurons responsible for presynaptically modulating the fSR**

The neurons which are presynaptic to the fSR have not yet been individually identified. A number of possible candidates can however, be proposed. It is likely that at least some of the GABA-IR processes presynaptic to the fSR are inhibitory flight interneurons. Eight intersegmental interneurons (301, 302, 501, 507, 511, 513, 520, 538) that are known to be involved in the locust flight system are GABA-IR (Robertson and Wisniowski, 1988). It is possible that some of the GABAergic interneurons that may be presynaptic to the fSR are activated by other proprioceptors associated with the wings. For example, the tegula (Burrows, 1976; Pearson and Wolf, 1988) and the

campaniform sensilla (Elson, 1987) are activated during the down-stroke of the wings and evoke IPSPs in the depressor motoneurons to prevent the wing from being depressed further. The IPSPs in the depressor motoneurons are the consequence of the proprioceptors connecting with interposing inhibitory interneurons which reverse the sign. Interneuron 515 and interneuron 511, which is known to be GABA-IR (Robertson and Wisniowski, 1988), receive inputs from the tegula and connect with depressor motoneurons (Robertson and Pearson, 1985). It is possible therefore, that one or more of these interposing inhibitory interneurons may also connect with the fSR to inhibit the release of ACh and reduce the response in the depressor motoneurons. The EM ICC shows that some of the GABA-IR processes make synaptic inputs onto processes postsynaptic to the fSR (e.g. depressor motoneurons) as well as the fSR itself (Figure 4.4C).

The glutamate-IR processes presynaptic to the fSR may be interneurons and/or motoneurons. Possible candidates include a group of ascending intersegmental interneurons, called the 404 interneurons, which receive sensory input from the forewings (Watson and Burrows, 1983) and are involved in initiating and maintaining locust flight (Pearson et al., 1985). The structure and position of this group of interneurons in the mesothoracic ganglion matches the structure and position of a glutamatergic bundle revealed in an ICC study by Watson and Seymour-Laurent (1993), indicating the 404 interneurons are glutamatergic. Another possibility is that they are flight motoneurons. Flight motoneurons, thought to be glutamatergic (Bicker et al., 1988; Watson and Seymour-Laurent, 1993), project to the dorso-lateral neuropil regions (Pflüger et al., 1988) that contain the branches of the fSR so are possible candidates for the glutamate-IR inputs to the fSR.

Possible sources of the processes presynaptic to the fSR that are not immunoreactive for either GABA or glutamate are the stretch receptor for the hindwing (hSR) and other classes of sensory neuron including several different types of mechanoreceptor from the wing and leg e.g. afferents from trochanteral hair plate and trochanteral campaniform sensillae. Octopaminergic DUM neurons, which have been shown to be involved in locust flight (Whim and Evans, 1988; Ramirez and Orchard, 1990; Stevenson and Meuser, 1997; Duch and Pflüger, 1999), may also be potential sources of the processes presynaptic to the fSR that are neither immunoreactive for GABA or glutamate. There is clear anatomical evidence that arborizations of all these neurons project into the same regions of the ganglionic neuropil as those of the fSR (Altman and Tyrer, 1977; Watson et al., 1991; Watson and England, 1991; Watson, 1984).

#### **4.4.3. Synaptic outputs from the fSR**

Thirty-eight percent of identified synapses on the fSR in *Locusta migratoria* in the GABA ICC study are from outputs to other neurons, and of these, 60% are located on the lateral field of branches. The lateral field of branches extends from the lateral edge of the dorsal neuropil throughout aLAC. This is the same region of the mesothoracic ganglionic neuropil into which flight motoneuron dendrites and other types of wing and leg sensory receptor terminations, including the hSR, project. The most common synaptic configuration (93%) of outputs from the fSR involved postsynaptic dyads in which both of the postsynaptic members are non-GABA-IR. Only 7% of the outputs from the fSR are to GABA-IR profiles. Outputs from the fSR to non-GABA-IR and GABA-IR processes are intermingled on all the branches. Likewise, there is no

segregation of inputs or outputs on particular branches of the fSR. Inputs and outputs are located on each of the three main fields of branches (lateral, medio-lateral, medial) frequently in close proximity to each other, suggesting that the presynaptic control of ACh release from the fSR is precisely targeted. The close proximity of input and output synapses on the fSR is similar to the arrangement of synapses reported in other types of insect sensory afferents (locust hSR, Altman et al., 1980; locust campaniform sensilla, Watson and England, 1991; locust hair plate afferents, Watson et al., 1991; locust FeCO afferents, Watson et al., 1993; locust prosternal filiform afferents, Watson and Pflüger, 1994; bushcricket and cricket auditory afferents, Hardt and Watson, 1999).

The processes which receive outputs from the fSR have not been identified in this study, but it is most likely that one of the non-GABA-IR members of the postsynaptic dyads is a forewing depressor flight motoneuron. Evidence to support this comes from electrophysiological studies (Burrows, 1975, Reye and Pearson, 1987), which demonstrate that the fSR makes excitatory monosynaptic connections with the basalar, dorsal longitudinal, and subalar depressor motoneurons which control the forewing (Burrows, 1975). Peters et al. (1985) estimated that each physiological connection of the hSR with the depressor motoneuron in the metathoracic ganglion, equivalent to BA1, is probably represented by as many as 600 anatomical synapses. Possible candidates for the second non-GABA-IR member of the postsynaptic dyad are (1) arborizations of the hSR which project into the mesothoracic ganglion and overlap almost totally with mesothoracic fSR projections (Altman and Tyrer, 1977), (2) other classes of sensory receptor from the wing and leg which project to aLAC e.g. afferents from trochanteral hair plate (Watson et al., 1991) and trochanteral campaniform

sensillae (Watson and England, 1991), and (3) interneurons which receive inputs from the fSR e.g. the excitatory flight interneurons 202, 503, 701 (Reye and Pearson, 1987).

Dyads at which one of the postsynaptic profiles is GABA-IR is likely to represent labelled dendritic processes of inhibitory flight interneurons which lie between the fSR and mesothoracic elevator motoneurons. In *Schistocerca gregaria*, spikes in the fSR evoke (in parallel with EPSPs in depressor motoneurons) IPSPs in tergosternal and tergocoxal elevator motoneurons, (Burrows, 1975). The IPSPs are of a longer latency than the EPSPs in depressor motoneurons, indicating that there are interposed interneurons which reverse the sign of the sensory signal. In *Locusta migratoria*, similar patterns of connection with flight motoneurons (Reye and Pearson, 1987). Of the 21 flight interneurons the fSR is reported to connect with (Reye and Pearson, 1987), 5 are known to be GABA-IR (Robertson and Wisniowski, 1988). These are the ipsilateral interneurons, 302, 501, 507, 520 and the contralateral interneuron 507.

#### **4.4.4. Networks involving GABA-IR neurons and the fSR**

Numerous networks involving GABA-IR inputs to the fSR were shown in this study. GABA-IR neurons were found presynaptic to terminal branches of the fSR and to non-GABA-IR dendrites postsynaptic to the fSR (Figure 4.4A,C), i.e., both the fSR and its non-GABA-IR postsynaptic member receive inputs from the same GABAergic neuron. Such an arrangement would allow both pre- and postsynaptic inhibition of the fSR afferent outputs, for example, at the fSR/BA1 synapse. This arrangement of connections and mechanism of modulation has also been reported at prosternal filiform afferent terminals in the locust *Schistocerca gregaria* (Watson and Pflüger, 1994), but

has never been observed at locust hair plate afferents (Watson et al., 1991) or campaniform sensilla afferents (Watson et al., 1991). This may in part underlie the differences previously noted (4.4.1.) in the proportion of GABA-IR inputs to each of these classes of afferents. In the latter examples (the hair plate and campaniform sensilla afferents), where there is no evidence from GABA ICC for inhibition postsynaptic to the afferent, the proportion of presynaptic inputs that are GABA-IR is high (93% and 72%, respectively). In the case of the prosternal filiform afferents and fSR afferent, where there is ICC evidence in support of pre- and postsynaptic inhibition, the proportion of GABA-IR inputs is much lower (51% and 43%, respectively).

Interactions were also found where GABA-IR neurons presynaptic to the fSR reciprocally connected with either non-GABA-IR neurons (Figure 4.3A) or other GABA-IR neurons (Figure 4.3B). An arrangement of reciprocal connections between GABA-IR neurons could represent a mechanism for mutual disinhibition (i.e., inhibition of one inhibitory interneuron by the other one) of inhibitory interneurons participating in synergistic pathways, for example, flight interneurons 301 and 501 (Burrows, 1996). Other examples of reciprocal synaptic contacts found in the study include reciprocal connections between two non-GABA-IR processes and reciprocal connections between non-GABA-IR processes and the fSR. Interestingly, however, no reciprocal contacts were found between GABA-IR processes and the fSR, indicating that either inputs from GABA-IR neurons to the fSR and outputs from the fSR to GABA-IR processes are located at sites distant from each other or that they are absent.

#### **4.4.5. Role of GABA and glutamate in presynaptic modulation**

The role of GABA in presynaptic inhibition of vertebrate and invertebrate sensory neurons has been extensively studied (see Watson, 1992a, Clarac and Cattaert, 1996). At arthropod afferent terminals, GABA-mediated presynaptic inhibition has so far proven to be chloride-dependent (Hue and Callec, 1983; Cattaert et al., 1992, 1994; Burrows and Laurent, 1993). Injection of chloride into cercal afferent terminals in the locust (which are known from ICC to receive GABA-IR inputs; Watson, 1990) causes a depolarising after potential, which is similar in size and duration to that evoked through stimulation of the afferent nerve (Boyan, 1988). Physiological studies on femoral chordotonal afferents also have indicated that GABA mediates depolarising IPSPs in their central terminals, which are due to an increase in chloride conductance (Burrows and Laurent, 1993). Injection of GABA into the neuropil close to the chordotonal afferent terminals causes a reduction in the excitability of the terminals, a reduction in the amplitude of the presynaptic afferent spikes, and a reduction in the EPSP amplitude evoked in the postsynaptic motoneuron by the afferent (Burrow and Laurent, 1993; Burrows and Matheson, 1994). The reduction in the excitability of the terminal far outlasts the change in chloride conductance, which suggests other unknown cellular events may also be involved in the inhibition (Burrows and Laurent, 1993). Presynaptic inhibition with a different ionic basis has been reported in the cockroach (Blagburn and Sattelle, 1987c), although the transmitters responsible are unknown. In this case, inhibition is caused by voltage-gated, delayed rectifying potassium channels instead of ligand-gated chloride channels.

Although there is evidence from EM ICC studies (Watson et al., 1991; Watson and Pflüger, 1994; Hardt and Watson, 1999) that glutamate is present in processes presynaptic to a number of insect sensory afferents, the role of glutamate in presynaptic modulation is still unknown. Glutamate has been shown to have both excitatory and inhibitory effects on invertebrate neurons. It depolarises and excites the locust FETi and flexor motoneurons (Sombati and Hoyle, 1984c; Parker, 1994) and hyperpolarises and inhibits the crayfish levator motoneuron (Pearlstein et al., 1998) and the cockroach fast coxal depressor motoneuron (Df) (Wafford and Sattelle, 1989). Pressure application of glutamate to locust flight motoneurons caused 46% of the motoneurons to hyperpolarise, which inhibited spontaneous action potentials, 38% of the motoneurons to depolarise and 16% of motoneurons produced a biphasic response (Dubas, 1990). Therefore, it is unclear if glutamate excites or inhibits the presynaptic terminals of the afferents which receive glutamate-IR inputs.

The results from this ICC study clearly demonstrate a widely distributed network of GABA-IR inputs to the fSR in *Locusta migratoria*. The results presented in Chapter 3 suggest that these GABA-IR neurons are activated via muscarinic receptors on their surface and that they are involved in modulating transmission at the fSR/BA1 synapse. Bath application of picrotoxin (a GABA antagonist) to the preparation causes a marked increase in the amplitude of electrically-evoked EPSPs recorded from BA1 but prevents the augmentation of EPSPs normally caused by muscarinic antagonists (Judge and Leitch, 1999c). Clearly then, complementary electrophysiological and neuropharmacological studies support the EM ICC study findings, which indicate a role for GABA presynaptic modulation at the fSR/BA1 synapse.



Nevertheless, modulation at this synapse appears to be more intricate. The results from a further EM ICC study shows that glutamate-IR processes are also presynaptic to the fSR, which indicates that the fSR is modulated by glutamate as well. In addition, other neurotransmitters released from the non-IR processes (shown by ICC in this study) as yet unidentified may also be involved in modulating the fSR/BA1 synapse.

## **Chapter 5. Octopaminergic modulation of cholinergic synaptic transmission between the fSR and BA1.**

### **5.1. INTRODUCTION**

Biogenic amines are a group of amines that are biologically active in both vertebrates and invertebrates. In the central nervous system (CNS) of both vertebrates and invertebrates, biogenic amines may act as neurotransmitters exciting or inhibiting neurons onto which they are released, and/or as neuromodulators. Octopamine, dopamine, and 5HT (also known as serotonin), are three such biogenic amines.

Octopamine is one of the most important biogenic amines in invertebrates (Evans, 1985; Orchard et al., 1993; Roeder, 1999). It was first discovered in the salivary glands of the octopus (Erspamer and Boretti, 1951), hence the name octopamine but has since been shown to be widely distributed in many other invertebrate species (Mercer et al., 1983; Lee and Wyse, 1991; Eckert et al., 1992; Spörhase-Eichmann et al., 1992; Stevenson et al., 1992; Elekes et al., 1993; Schneider et al., 1993; Monastirioti et al., 1995; Stevenson and Spörhase-Eichmann, 1995). It has been suggested that octopamine is involved in the modulation of every physiological process in invertebrates studied so far (Roeder, 1999). It is an analogue of the vertebrate monamine noradrenaline, possessing one of the two hydroxyl groups on the aromatic ring, and interestingly, the roles octopamine plays in numerous invertebrate preparations are similar to those played by adrenaline and noradrenaline in vertebrates; it has been reported to act as a neurohormone, a neuromodulator and a neurotransmitter (Orchard, 1982; Roeder, 1999). Its action has been shown to be widespread, ranging from its effects on muscles

in the periphery (Candy, 1978; Evans, 1981; Whim and Evans, 1988) to its effects on neurons in the CNS (Sombati and Hoyle, 1984 a,b; Claassen and Kammer, 1986; Stevenson and Kutsch, 1987; Ramirez and Pearson, 1991). Its effects are also diverse ranging from locomotory behaviours such as flight (Orchard et al., 1993) to more complex functions such as learning and memory (Mercer and Menzel, 1982; Hammer, 1993).

The octopaminergic modulation of numerous different behaviours has been extensively studied, but no other behaviour has been studied in as much detail as locust flight (Orchard et al., 1993). Flight like other behaviours has to be continuously modulated to suit the changing environment, so it is not surprising that octopamine plays such an important role here. The functions of octopamine in flight include it acting as a neurohormone, a neurotransmitter and a neuromodulator. Neurohormones are released into an animal's circulation e.g. the locust haemolymph enabling them to act on distant targets. Evidence that octopamine acts as a neurohormone in locust flight was demonstrated in a study by Goosey and Candy (1980). They showed that the concentration of octopamine in the haemolymph of adult locusts increases rapidly after the onset of flight and then begins to decrease after 10 minutes, indicating it is involved in the early period of flight. It is believed that one of the reasons octopamine is released into the haemolymph after the onset of flight is to regulate the metabolism needed for such an energy-demanding activity. Energy substrates from the fat body, which is the major storage site in insects, are mobilised and transported via the haemolymph to the flight muscles. It appears that octopamine regulates the release of lipids (diacylglycerols) from the fat body during the first few minutes of flight, by acting on the fat body itself (Van Heusden et al., 1984; Orchard and Lange, 1985).

Additionally, octopamine acting as a neurotransmitter appears to be involved in the mobilisation of lipids from the fat body during more prolonged flight. The two adipokinetic hormones, AKH I and AKH II, are released after approximately 10 minutes of flight from neurosecretory cells in the glandular lobe of the corpus cardiacum into the haemolymph (Orchard and Lange, 1983; Orchard 1987). The function of these hormones is to mobilise lipids from the fat body (Orchard, 1987). The release of these hormones can be evoked either by the electrical stimulation of the nervi corporis cardiaci II (NCC II) which projects into the glandular lobe of the corpus cardiacum or the application of octopamine to the glandular lobes (Orchard et al., 1983; Orchard and Lange, 1983; Orchard, 1987). Further evidence that the synapse between the NCC II and neurosecretory cells is octopaminergic was demonstrated when the octopamine-induced elevation of cAMP in the neurosecretory cells and the release of the AK hormones was blocked by reported insect octopamine receptor antagonists (Orchard and Loughton, 1981; Orchard et al., 1983; Pannabecker and Orchard, 1986). Therefore, octopamine regulates the mobilisation of the energy substrates to the flight muscles, by acting both as a neurohormone and a neurotransmitter.

The flight muscles themselves are also known to be under the modulation of octopamine. Octopamine not only stimulates the oxidative metabolism in flight muscles (Candy, 1978) but also modulates flight muscle contraction (Whim and Evans, 1988). Stimulation of an identified octopaminergic dorsal unpaired median (DUM) neuron, DUMDL, or application of octopamine, increases the amplitude of twitch tension in the dorsal longitudinal flight muscles, which causes an increase in the amount of force generated (Whim and Evans, 1988). Additionally, octopamine causes the twitch duration of the dorsal longitudinal flight muscles to decrease at low temperatures but is



less effective at high temperatures. This temperature-dependent effect is suggested to be an energy saving adaptation, to allow better contraction synchronisation at the onset of flight, when the body temperature is low.

The actual synchronisation of the contraction of flight muscles to produce co-ordinated wing movements in the locust is controlled by the flight motor pattern. The central neurons, which generate this rhythmic activity and the proprioceptors that regulate the rhythm are also reported to be modulated by octopamine. Modulation of the proprioceptors by octopamine allows the sensory information carried by them to be regulated before it is integrated into the flight motor pattern. The fSR is a proprioceptor which relays information about the position of the forewing during upstroke and has been shown to reset flight rhythm (Pearson et al., 1983; Reye and Pearson, 1988).

Raising the levels of octopamine in the haemolymph to a concentration equal to that measured during the first few minutes of flight, enhances the responses of the fSR to wing movements (Ramirez and Orchard, 1990). Both the number and frequency of spikes recorded from the peripheral nerve increase. DUM neurons are implicated as the source of the octopamine responsible for the modulation of the fSR (Ramirez and Orchard, 1990). Stimulation of the nerves which contain DUM neuron axons enhances the response of the fSR and this effect is blocked by injecting phentolamine, a reported insect octopamine receptor antagonist, into the haemolymph (Ramirez and Orchard, 1990). Octopamine has also been reported to cause an increase in the responsiveness of another flight sensory pathway. Electrical stimulation of tegula afferents, which monitor wing position during downstroke, evokes excitatory postsynaptic potentials (EPSPs) in flight elevator motoneurons. The amplitude of the evoked EPSPs recorded from the elevator motoneurons increase following octopamine application (Ramirez

and Pearson, 1991). The increase in the excitatory response of the elevator motoneurons is due to the increased excitability of interneurons interposed between the tegula and the flight motoneurons. The intrinsic changes in these interneurons, induced by octopamine were studied in detail by Ramirez and Pearson (1991). It was shown that injection of depolarising current into these interneurons or synaptic input from the tegula in the presence of octopamine resulted in voltage-dependent plateau potentials. The injection of longer depolarising current pulses evoked rhythmic bursting in various interneurons in the presence of octopamine. The frequency of this bursting is similar to the rhythm seen in the same interneurons in deafferented flying locusts, indicating octopaminergic modulation of these interneurons may play a role in generating the flight motor pattern. Indeed, injection of octopamine into the specific neuropil area thought to contain these neurons, induces flight motor activity in locusts (Sombati and Hoyle, 1984b; Stevenson and Kutsch, 1987). Octopamine injection into the dorso-lateral regions of the locust metathoracic ganglion induced rhythmic motor activity consisting of cycles of alternating elevator/depressor excitation (Sombati and Hoyle, 1984b). Flight motoneurons on both the ipsilateral and contralateral side of the ganglion were synchronously activated in a pattern which resembled the flight motor activity. Similar results were found in the moth *Manduca sexta*; octopamine injection into the lateral regions of the mesothoracic ganglion in the moth induced a flight motor pattern (Claassen and Kammer, 1986). The flight motor pattern induced in the moth was dependent on sensory input, which was also shown to be modulated by octopamine (Claassen and Kammer, 1986). The synchronous activation of the flight motoneurons and the time relationships between them induced by octopamine in both locusts and moths was similar to the natural flight pattern, which indicates octopamine has an

important role in the generation and regulation of the neural circuitry that controls flight.

These studies show that octopamine plays a role in nearly all aspects of locust flight; it is involved in regulating the energy metabolism needed for flight, it modulates the flight muscles, it is capable of generating a flight motor pattern and it modulates the proprioceptors which carry the sensory information needed to regulate the flight motor pattern. One reason suggested for a widespread action of a neuromodulator in a behaviour such as flight is to change the arousal state of the animal. The release of a neuromodulator such as octopamine and the combination of its widespread actions could shift the behavioural state of the animal from one level to another. A comparative example of this would be the adrenaline-induced 'fight or flight' behaviour in vertebrates.

Although the role of octopamine in locust flight has been studied in great detail and used as a model to study the neuromodulation of behaviour in invertebrates, the study of other behaviours that are also modulated by octopamine e.g. walking have generated much interesting information. In much the same way that octopamine modulates nearly all aspects of flight, octopamine is reported to modulate numerous neurons and muscles involved in walking (Evans, 1981; Sombati and Hoyle, 1984 a,b; Ramirez et al., 1993). These include the stick insect femoral chordotonal organ neurons that code the position of the leg joint (Ramirez et al., 1993), and also the locust SETi motoneuron and the locust extensor-tibiae muscle (Evans, 1981). The source of the octopamine responsible for modulating the extensor-tibiae muscle and the SETi motoneuron has been identified as an octopaminergic DUM neuron, DUMETi (Evans and O'Shea, 1977). As well as

octopamine acting on peripheral neurons, it is also reported to modulate neurons within the CNS that control leg movements (Sombati and Hoyle, 1984 a,b). Injection of octopamine into specific regions of the locust metathoracic ganglion dishabituates and sensitises flexor motoneurons to synaptic inputs from fast extensor tibiae motoneurons (Sombati and Hoyle, 1984a) and induces rhythmical flexor bursting, the rate of which resembles fast walking (Sombati and Hoyle, 1984b). Evidence of the involvement of DUM neurons in the modulation of these motoneurons was demonstrated by stimulating an identified DUM neuron, DUM 3(a,b),4 (Sombati and Hoyle, 1984a). Stimulation of DUM 3(a,b),4 dishabituated and sensitised the inputs to an identified flexor motoneuron and evoked rhythmic extension/flexion of the metathoracic tibiae.

Escape behaviours have also been reported to be modulated by octopamine (Glanzman and Krasne, 1983; Goldstein and Camhi, 1991). Octopamine enhances the responsiveness of lateral giant neurons involved in the escape reaction in the crayfish (Glanzman and Krasne, 1983) and the responsiveness of interneurons that modulate the cockroach escape response to cercal stimulation (Goldstein and Camhi, 1991). Another behaviour controlled by a motor pattern, reported to be modulated by octopamine, is oviposition. Octopamine inhibits both the neural control of rhythmical oviposition digging in female locusts (Sombati and Hoyle, 1984b) and the contractions of oviduct muscle (Lange and Tsang, 1993).

Octopaminergic modulation of behaviours is not just restricted to regulation of motor control and locomotory behaviours. Octopamine has been shown to modulate complex sensory processes such as vision (Kass and Barlow, 1984; Erber et al., 1993; Bacon et al., 1995; Stern et al., 1995), and olfaction (Mercer and Erber, 1983). It has been shown



to play a role in learning and memory (Mercer and Menzel, 1982; Dudai et al., 1987; Hammer, 1993). There is evidence that octopamine modulates the visual input to the brain in the horseshoe crab, *Limulus polyphemus* (Kass and Barlow, 1984) and in the honeybee, *Apis mellifera* (Erber et al., 1993) by increasing the sensitivity of the retina. Octopamine also appears to increase the sensitivity of neurons involved in visual output (Bacon et al., 1995; Stern et al., 1995). Stimulation of identified octopaminergic protocerebral medulla 4 neurons in the locust brain dishabituates the descending contralateral movement detector neuron, which carries information from the visual system in the locust (Bacon et al., 1995; Stern et al., 1995). Higher order processing of sensory information is also modulated by octopamine. Mushroom bodies are neuropil regions in the insect brain that are regarded as the invertebrate equivalent of the hippocampus, as they are implicated in learning and memory. Light-evoked potentials recorded from the mushroom bodies of the honey bee, *Apis mellifera*, increase by 50% following the application of octopamine (Mercer and Erber, 1983). In contrast, potentials evoked by olfactory stimuli following the application of octopamine decrease in some individuals and increase in others (Mercer and Erber, 1983). The differences in the action of octopamine may be associated with its role in learning and memory. Injection of octopamine into the brain enhances the responsiveness of honey bees to previously ineffective olfactory stimuli i.e. it has a sensitising effect (Mercer and Menzel, 1982). Additionally, an identified octopaminergic neuron in the honey bee brain, the VUMmx1 (ventral unpaired median cell of the maxillary neuromere) is involved in reinforcing the association between conditioned and unconditioned stimuli in olfactory learning (Hammer, 1993). In the fruit fly, *Drosophila* on the other hand, octopaminergic agonists decrease olfactory learning (Dudai et al., 1987).

The importance of octopamine in modulating behaviour is clearly evident from the studies described so far. Its action is widespread and diverse and its multifunctional role in a number of behaviours has led to the suggestion that it is released to shift the behavioural state of the animal from one level to another. Recently the receptors, which mediate the action of octopamine in invertebrates, have received a lot of attention. This is because the receptors are the site at which octopamine mediates its action and they are potentially ideal targets for insecticides as they do not have direct counterparts in vertebrates.

All invertebrate octopamine receptors reported so far are G-protein-coupled receptors, but they are coupled to a number of different second messenger systems. The majority of octopamine receptors are coupled to the enzyme adenylate cyclase. In fact, the first identification of octopamine receptors was done so on the basis of their positive coupling to adenylate cyclase (Nathanson and Greengard, 1973; Nathanson, 1979). Evidence of octopamine receptors positively coupled to adenylate cyclase have been reported in many preparations. These include the CNS of the cockroach (Nathanson and Greengard, 1973; Orr et al., 1991), fruit fly (Uzzan and Dudai, 1982), tobacco hornworm (Orr et al., 1991), locust (Orchard et al., 1983; Roeder and Nathanson, 1993), cockroach haemocytes (Orr and Hollingworth, 1990), the firefly lantern (Nathanson, 1979, 1985), locust extensor-tibiae motoneurons and muscles (Evans, 1984a,b), horseshoe crab photoreceptors (Kaupp et al., 1992), and locust lateral oviducts (Lange and Tsang, 1993). A cloned octopamine receptor from the snail, *Aplysia* is also reported to be coupled to adenylate cyclase (Li et al., 1994). In other preparations octopamine receptors are coupled to second messenger pathways which involve calcium. Octopamine receptors on cockroach haemocytes, as well as being

**Table 5.1. Rank order of affinities of agonists and antagonists for the insect octopamine receptor classes**

Receptor class	Agonists	Antagonists	Reference	Preparation
Octopamine 1	Tola > naph = DL-octo	Phent >> mian > meto	Evans (1981)	locust extensor-tibiae muscle
Octopamine 2A	Naph >> tola = DL-octo	Meto = mian = phent	Evans (1981)	locust slow extensor-tibiae motoneuron
Octopamine 2B	Naph = tola = DL-octo	Phent > meto >> mian	Evans (1981)	locust extensor-tibiae muscle
Octopamine 3	Naph = DL-octo = tola	Mian > phent >> meto	Roeder (1992)	locust CNS

The affinities of the agonists and antagonists are from Evans (1981) for the the octopamine 1, 2A and 2B receptor class and from Roeder (1992) for the octopamine 3 receptor class. = represents a difference of less than a factor of 10, > represents a difference of a factor of 10 or more and >> represents a difference of a factor of 50 or more. Meto = metoclopramide, mian = mianserin, naph = naphazoline, DL-octo = DL-octopamine, phent = phentolamine.

coupled to adenylate cyclase (Orr and Hollingworth, 1990), are positively coupled to inositol triphosphate (IP<sub>3</sub>), which leads to an increase in intracellular calcium from internal stores (Baines and Downer, 1994). Cloned *Drosophila* tyramine/octopamine receptors expressed in mammalian cell lines also increase intracellular calcium levels when stimulated by octopamine, but interestingly inhibit adenylate cyclase activity when activated by tyramine (Arakawa et al., 1990; Saudou et al., 1990; Robb et al., 1994). This indicates that a particular receptor may be coupled to alternative second messenger pathways depending upon the agonist by which it is activated.

Vertebrate  $\alpha$ -adrenergic receptors, like octopamine receptors, are G-protein-coupled. Additionally, octopamine receptors and  $\alpha$ -adrenergic receptors have similar pharmacological profiles. Many  $\alpha$ -adrenergic agonists and antagonists are reported to act at insect octopamine receptors (Evans, 1981; Roeder, 1992). Although the agonists and antagonists reported to act at octopamine receptors are unfortunately not completely selective for particular receptor classes, they do have different affinities and have been used to identify different insect octopamine receptors (see Table 5.1). In the locust extensor-tibiae-neuromuscular preparation a number of different octopamine receptor classes have been identified (Evans, 1981). The octopamine<sub>1</sub> receptors are located on the extensor tibiae muscle fibres and mediate the slowing of the myogenic rhythm of contraction. The octopamine<sub>2A</sub> receptors are thought to be located on the presynaptic terminals of the SETi motoneuron and modulate transmitter release from the presynaptic terminals of SETi. The octopamine<sub>2B</sub> receptors are also located on the muscle fibres and increase the relaxation rate of twitch tension. Octopamine receptors that possess the same pharmacology as those characterised in the locust extensor-tibiae neuromuscular preparation have been identified in other insect preparations.

Octopamine<sub>2</sub> receptors, which are positively coupled to adenylate cyclase, have been identified in the firefly lantern (Nathanson, 1985), the locust lateral oviducts (Lange and Tsang, 1993) and the locust corpora cardiaca (Pannabecker and Orchard, 1986). Binding studies with <sup>3</sup>H-octopamine have identified another octopamine receptor with a different pharmacological profile (Roeder, 1990; Roeder and Gewecke, 1990). This new octopamine receptor is only found in the CNS and named the octopamine<sub>3</sub> receptor (Roeder, 1992, 1995).

The very presence of octopamine receptors in the CNS supports a modulatory role for octopamine in the CNS, as described above. As octopamine is known to modulate centrally located neurons and appears to be involved in all aspects of locust flight, it is a strong candidate as a potential neuromodulator of the centrally located cholinergic synapse between the fSR and BA1. Octopaminergic modulation of cholinergic synaptic transmission between identified neurons has been reported previously, only in a study of cholinergic synaptic transmission between the giant interneurons and thoracic interneurons involved in the cockroach escape response, where it was reported to increase EPSP amplitude (Casagrand and Ritzmann, 1992a,b). However, the location and identity of the receptors, which mediated this modulation were not examined. Therefore, the aims of the experiments conducted in this chapter were; a) to establish if octopamine does modulate cholinergic synaptic transmission between the fSR and BA1, b) to determine if octopamine modulates the synapse by acting on the postsynaptic neuron BA1, and/or presynaptic neurons, c) to characterise the receptors octopamine is acting at and d) to examine the potential sources of octopamine. Preliminary accounts of this work have been published in abstract form (Judge and Leitch, 1999a, 2000).

## **5.2. MATERIALS AND METHODS**

### **5.2.1. Electrophysiology**

Electrophysiology was used to investigate the possibility that octopamine modulates synaptic transmission between the fSR and BA1. The methods used to evoke synaptic transmission between the fSR and BA1 and elicit cholinergic responses in BA1 are described in detail in Chapter 2.2.

The effects of following compounds were tested: metoclopramide; mianserin; naphazoline; DL-octopamine; phentolamine; tolazoline. All reagents were purchased from Sigma (Poole, U.K.). The compounds were applied at concentrations shown to be effective in other insect preparations.

The effects of the compounds on the electrically-evoked EPSPs and ACh responses recorded from BA1 were calculated as percentage changes in control amplitude. All percentage means and standard errors of percentage means reported in the results have been arc-sine transformed unless otherwise stated (Chapter 2.2.5). A two-tailed paired t-test was used to assess the significance of the percentage changes between control amplitudes and amplitudes in the presence of compounds. A two-tailed unpaired t-test was used to assess the significance of the percentage changes between the response amplitudes in the presence of different compounds.

### 5.2.2. Confocal microscopical immunocytochemistry

Confocal microscopical immunocytochemistry was used to determine if octopamine is present in the neuropil region where it could potentially modulate the fSR/BA1 synapse. The general method used to immunolabel transverse slices for confocal microscopy has been described in Chapter 2.6. However the anti-octopamine antibody is conjugated to the fixative [1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDCDI)], so a modification of the method described by Karhunen et al. (1993) was used.

The mesothoracic ganglia from adult *Locusta migratoria* were removed and fixed overnight at 4°C in 4% EDCDI (Sigma, Poole, U.K.) dissolved in 0.1M Sorensens phosphate buffer (Appendix 4). After fixation, ganglia were sliced into transverse sections (75-100 µm thick) which were kept in sequence to aid the identification of the level at which each slice had been taken through the mesothoracic ganglion. After being washed in 0.1M Sorenson's phosphate buffer followed by BSA/Tris/X (pH 7.4), the slices were incubated for 1 hour in 2% normal goat serum dissolved in BSA/Tris/X. The slices were then incubated overnight at 4°C in rabbit anti-octopamine antiserum (Chemicon, Temecula, U.S.A.) diluted 1:500 with BSA/Tris/X. The anti-octopamine antibody was obtained from rabbits after immunisation with a DL-octopamine-EDCDI conjugate (Karhunen et al., 1993). The slices were then labelled with Rhodamine Red-labelled goat anti-rabbit antibody diluted 1:200 (Jackson ImmunoResearch Laboratories, West Grove, U.S.A.) before being mounted and examined on the confocal microscope (Chapter 2.6).

Positive and negative controls were performed to test the specificity of the labelling. The distribution of labelling in the slices incubated with the anti-octopamine antibody was examined to determine if the antibody labelled known octopamine-immunoreactive (OA-IR) neuropil regions and tracts reported by Stevenson et al. (1992). In negative control tests, the primary antibody was omitted and replaced with phosphate buffer. The negative controls did not show any immunolabelling.

### **5.3. RESULTS.**

#### **5.3.1. Effects of octopamine on electrically-evoked EPSPs**

The possibility that the biogenic monamine octopamine may modulate cholinergic synaptic transmission between the sensory afferent, fSR and the motoneuron, BA1 was investigated. The effects on the electrically-evoked EPSPs of bath application of DL-octopamine ( $10^{-6}$ - $10^{-3}$  M) were examined.

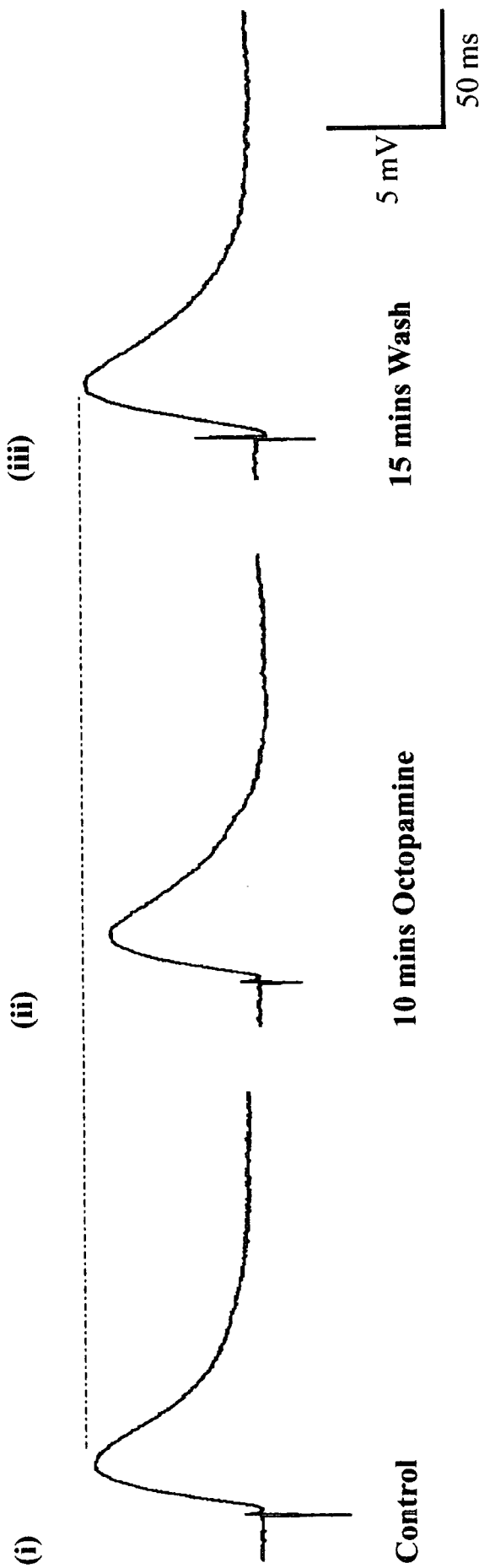
Under control conditions electrical stimulation of the fSR evoked EPSPs in BA1 with a mean amplitude of  $5.79 \pm 0.49$  mV (mean  $\pm$  SEM;  $n = 12$ ) and a duration of  $115 \pm 4$  ms (mean  $\pm$  SEM;  $n = 12$ ) (Figure 5.1i). Following the bath application of DL-octopamine ( $10^{-4}$  M) both the amplitude and duration of the electrically-evoked EPSPs recorded from BA1 decreased (Figure 5.1ii). The effects of octopamine on the EPSPs were reversed by washing the preparation with drug-free saline (Figure 5.1iii).

Bath application of DL-octopamine ( $10^{-4}$  M) caused the EPSP amplitude recorded from BA1 to decrease by a maximum mean percentage of 15.6 % (+SEM 1.6; -SEM 1.6;  $p <$



**Figure 5.1. Effects of Octopamine ( $10^{-4}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) DL-Octopamine ( $10^{-4}$  M) caused a decrease in the EPSP amplitude and duration. (iii) This decrease was reversible after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

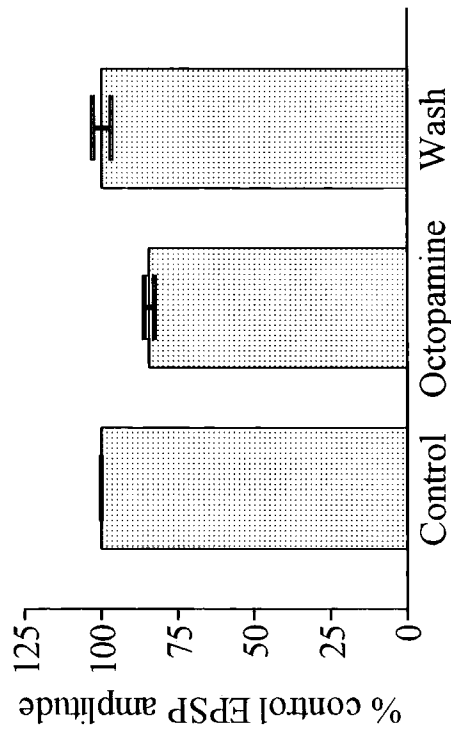
**Figure 5.1. Effects of Octopamine ( $10^{-4}$  M) on electrically-evoked EPSPs.**



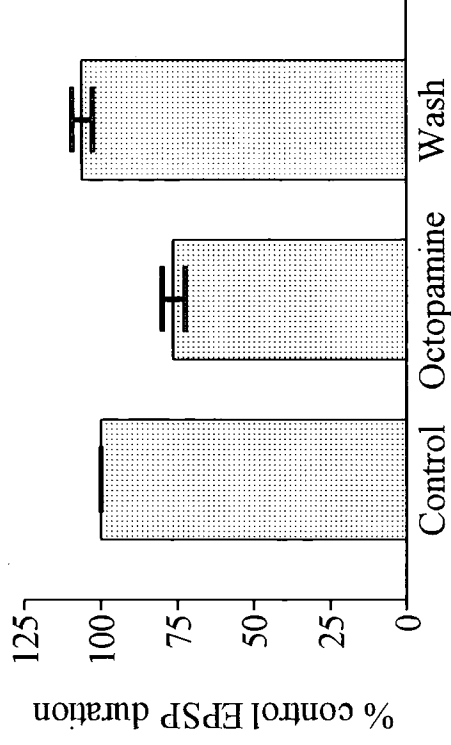
0.0001;  $n = 12$ ) (Figure 5.2A). The duration of the EPSP decreased following the application of DL-octopamine ( $10^{-4}$  M) by a maximum mean percentage of 23.7 % (+SEM 3.7; -SEM 3.9;  $p < 0.0001$ ;  $n = 12$ ) (Figure 5.2B). The decrease in both the mean EPSP amplitude and duration reversed to control levels after washing the preparation with drug-free saline.

The effects of DL-octopamine on the electrically-evoked EPSP amplitude began within 5 minutes following DL-octopamine application and had generally reached a maximal effect within 10 minutes (Figure 5.3). The EPSP amplitude generally returned to control levels, 15-25 minutes after the start of washing with drug-free saline.

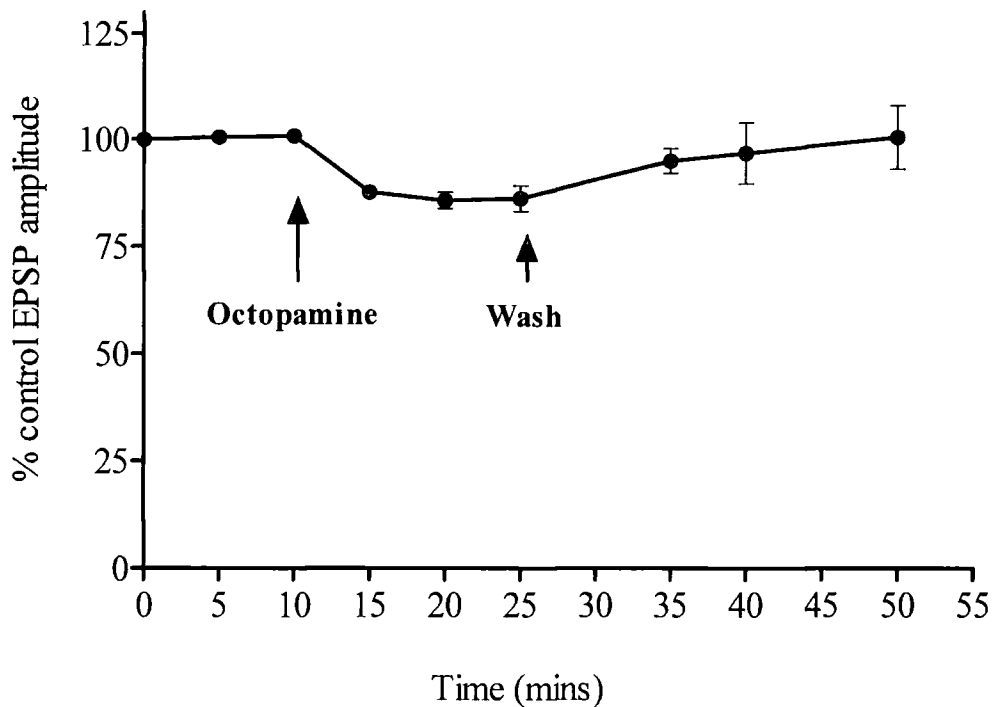
To determine if the inhibitory action of DL-octopamine ( $10^{-4}$  M) on the electrically-evoked EPSPs was due to a change in the membrane conductance of BA1, both the membrane potential and the input resistance of BA1 were monitored. BA1 depolarised by 1-6 mV within the first 5 minutes following DL-octopamine application, but the membrane potential of BA1 generally returned to resting potential levels within 10 minutes. As the EPSP amplitude and duration were still depressed at 10-15 minutes following DL-octopamine application it appears that the decrease in EPSP amplitude and duration is not the result of changes in the membrane potential of BA1. Also, the input resistance of BA1 was not significantly different ( $p = 0.48$ ) in the presence of DL-octopamine. These observations indicate that DL-octopamine may cause immediate changes in the membrane conductance on BA1 but the slower effects of DL-octopamine on the electrically-evoked EPSPs appear to be due to a mechanism other than a change in membrane conductance.



**Figure 5.2A. Maximum effect of Octopamine ( $10^{-4}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BAL in the absence (Control) and presence of octopamine and following wash ( $n = 12$ ). The amplitude is given as a percentage of the control amplitude.



**Figure 5.2B. Maximum effect of Octopamine ( $10^{-4}$  M) on EPSP duration.** Graphical representation of the pooled data from 12 experiments showing the duration of the EPSPs recorded from BAL in the absence (Control) and presence of octopamine and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.



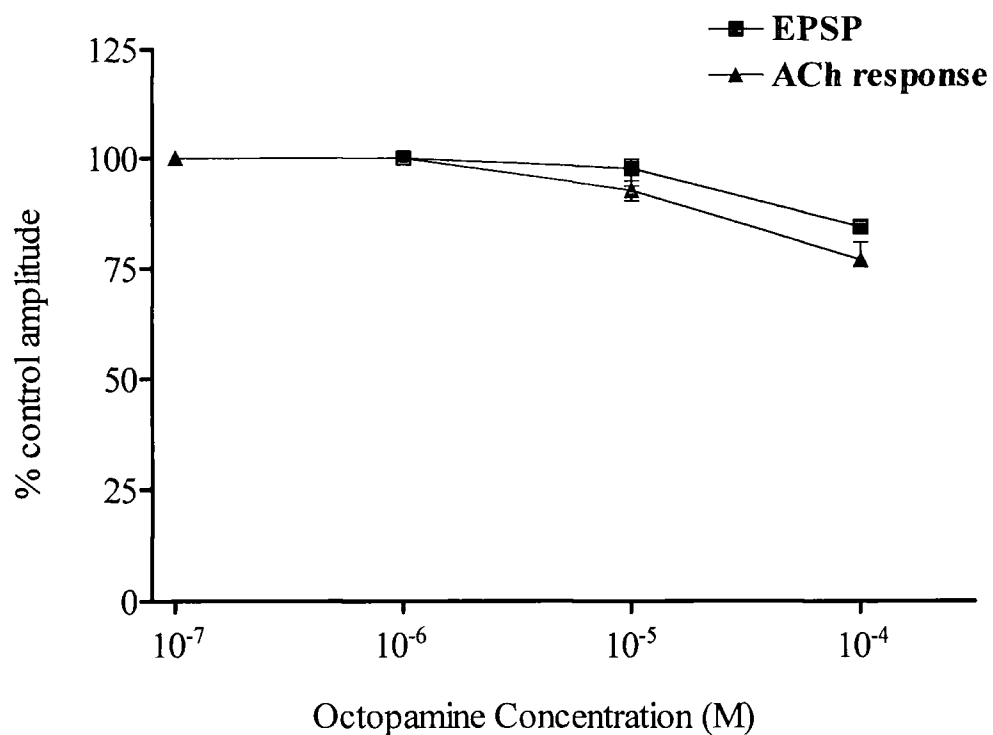
**Figure 5.3. Effect of Octopamine ( $10^{-4}$  M) on EPSP amplitude over time.** Groups of 8 successive electrically-evoked EPSPs were recorded from BA1 at 5 minute time intervals in the absence (control) and presence of DL-octopamine and following wash. The EPSP amplitude is given as a percentage of the control amplitude and the mean ( $\pm$  SEM) amplitude for 12 experiments is plotted for each 5 minute interval. The EPSP amplitude began to decrease within 5 minutes following octopamine application (indicated by long arrow) and had reached its maximum effect within 10 minutes. After the start of washing (indicated by short arrow) the EPSP amplitude began to increase, returning to control levels after 15-25 minutes.

The action of DL-octopamine on electrically-evoked EPSPs was found to be concentration dependent. The threshold concentration at which DL-octopamine had a significant effect on EPSPs recorded from BA1 was  $10^{-4}$  M (Figure 5.4). Bath application of DL-octopamine at concentrations of  $10^{-5}$  M ( $n = 5$ ) and  $10^{-6}$  M ( $n = 2$ ) had no significant effect on the EPSP amplitude or duration. Application of DL-octopamine at concentrations higher than  $10^{-3}$  M caused BA1 to depolarise to spiking threshold. This resulted in spontaneous action potentials and in the electrical stimulation of the fSR evoking action potentials rather than EPSPs in BA1. This meant it was difficult to assess the effects on the EPSP amplitude and duration recorded from BA1 of DL-octopamine at this concentration.

To summarise, the application of DL-octopamine caused a reversible decrease in both the amplitude and duration of the EPSPs recorded from BA1. The inhibitory action of DL-octopamine on the EPSPs was concentration dependent, causing a decrease in the amplitude and duration at  $10^{-4}$  M. This decrease in EPSP amplitude and duration does not appear to be due to changes in the membrane conductance of BA1. These results indicate that firstly, the monamine, octopamine is potentially capable of inhibiting cholinergic synaptic transmission between the fSR and BA1 and secondly, octopamine appears to mediate its inhibitory action on the fSR/BA1 synapse via mechanisms other than changes in the membrane conductance of BA1.

### **5.3.2. Effects of octopamine on ACh responses**

DL-Octopamine could inhibit synaptic transmission at the fSR/BA1 synapse by acting on presynaptic neurons and/or on the postsynaptic neuron BA1. If it exerts its inhibitory



**Figure 5.4. Concentration dependent effects of Octopamine.** Graph showing the mean ( $\pm$  SEM) amplitudes of EPSPs and ACh responses recorded from BA1 in the presence of different concentrations of octopamine. The amplitude is given as a percentage of the control amplitude.

action by acting on presynaptic neurons it could reduce the amount of ACh released from the fSR terminals, leading to a decrease in the EPSPs recorded from BA1. DL-Octopamine may also, however, cause a suppression of the response of the postsynaptic neuron BA1 to ACh. The possibility that DL-octopamine affects the cholinergic response of BA1 was investigated. Brief pulses of ACh were directly pressure-applied onto the cell body of BA1 resulting in depolarising responses of similar amplitudes. The effects of DL-octopamine ( $10^{-7}$  -  $10^{-4}$  M) on these postsynaptic cholinergic responses were then examined.

Under control conditions pressure application of brief (100-200 ms) pulses of ACh to the cell body of BA1 resulted in depolarising responses with a mean amplitude of  $20.75 \pm 1.75$  mV (mean  $\pm$  SEM;  $n = 10$ ). Following the bath application of DL-octopamine ( $10^{-4}$  M) the amplitude of the ACh responses recorded from BA1 decreased (Figure 5.5A). The effects of DL-octopamine on the ACh responses reversed after washing the preparation with drug-free saline.

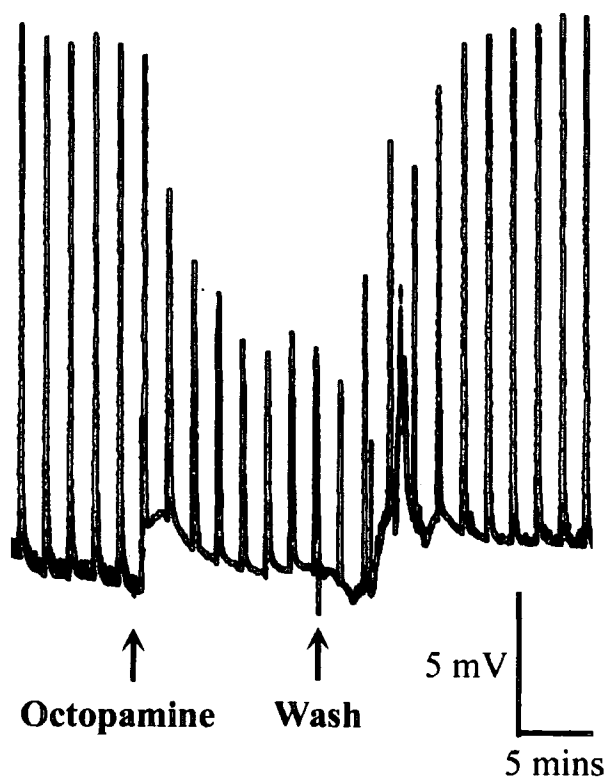
The amplitude of the ACh responses recorded from BA1 began to decrease within 1 minute following the application of DL-octopamine ( $10^{-4}$  M) and had generally reached a maximum decrease within 10-15 minutes following application. DL-Octopamine ( $10^{-4}$  M) caused a mean maximum decrease of 23 % (+SEM 3.9; -SEM 1.2;  $p < 0.0001$ ;  $n = 10$ ) in the amplitude of the ACh responses recorded from BA1 (Fig. 5.5B). The amplitude of the ACh responses returned to control levels within 15 minutes after the start of washing with drug-free saline.



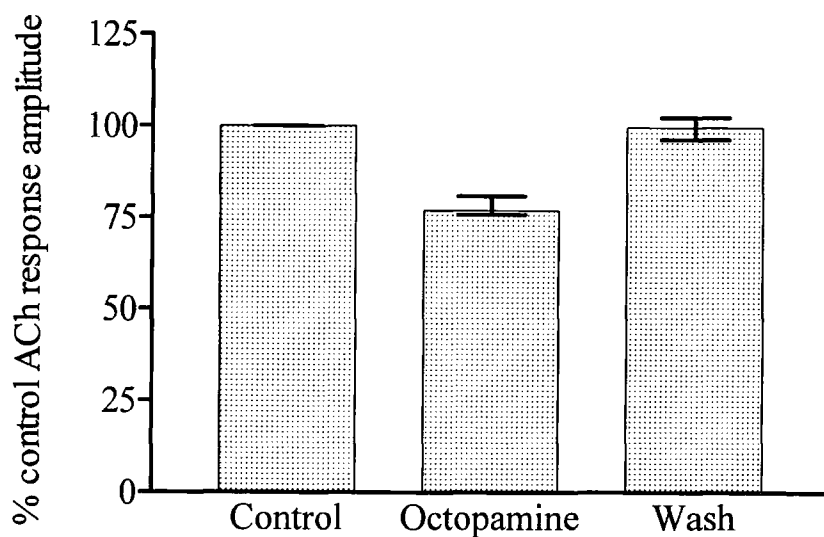
**Figure 5.5A. Effects of Octopamine ( $10^{-4}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses (100-200 ms) of acetylcholine (ACh) onto the cell body of BA1, resulted in depolarising responses in BA1. Following the application of DL-octopamine ( $10^{-4}$  M) the amplitude of the ACh responses decreased. The amplitude of the ACh responses returned to control levels after washing. Note that spontaneous activity recorded from BA1 is inhibited in the presence of octopamine and returns after washing.

**Figure 5.5B. Maximum effect of Octopamine ( $10^{-4}$  M) on ACh response amplitude.** Graphical representation of the pooled data from 10 experiments showing the amplitude of the ACh responses recorded from BA1 in the absence (control) and presence of octopamine and following wash. The mean ( $\pm$  SEM) amplitude is given as a percentage of the control amplitude.

**Figure 5.5A. Effects of Octopamine ( $10^{-4}$  M) on ACh responses recorded from BA1.**



**Figure 5.5.B. Maximum effect of Octopamine ( $10^{-4}$  M) on ACh response amplitude.**



The inhibitory action of DL-octopamine on ACh responses recorded from BA1 was found to be concentration dependent. The threshold concentration at which DL-octopamine had a detectable effect on ACh response amplitudes was approximately  $10^{-5}$  M (Figure 5.4). Following the bath application of DL-octopamine ( $10^{-5}$  M) the ACh response amplitude decreased by a mean percentage of 7.3 % (+SEM 2.1; -SEM 2.5;  $p = 0.0295$ ;  $n = 3$ ). The decrease in ACh response amplitude reversed after washing the preparation with drug-free saline. Bath application of DL-octopamine at concentrations of  $10^{-6}$  M ( $n = 2$ ) and  $10^{-7}$  M ( $n = 1$ ) had no detectable effect on the ACh response amplitude.

To determine if the decrease in ACh response amplitude following the application of DL-octopamine was due to a change in the membrane conductance of BA1, both the membrane potential and the input resistance of BA1 were monitored throughout the experiments. The input resistance of BA1 did not change significantly ( $p = 0.53$ ) with the decrease in ACh response amplitude but in all experiments the membrane potential of BA1 did. BA1 depolarised by 0.5-6 mV within the first 2 minutes following the application of DL-octopamine ( $10^{-5}$  -  $10^{-4}$  M) (see Figure 5.5A). The membrane potential of BA1, however, generally returned to resting potential levels within 10 minutes of octopamine application at which time the ACh response amplitude was still depressed. In certain experiments the membrane potential of BA1 did not repolarise to resting potential levels after depolarising. To determine if the decrease in ACh response amplitude was due to BA1 depolarising, brief pulses (5 ms) of hyperpolarising current (0.1 - 0.5 nA) were injected at rate of 100 Hz through the intracellular recording electrode into BA1. This hyperpolarising current repolarised the cell to approximate resting potential levels and the amplitude of ACh responses recorded under these

conditions was monitored. In these cases the ACh response amplitude was approximately the same value as the ACh response amplitudes recorded before hyperpolarising current was injected. These observations indicate that the effects on the ACh responses following the application of DL-octopamine are not due to changes in the membrane conductance of BA1.

An additional observation during these experiments was the effect the application of DL-octopamine had on spontaneous activity. In some preparations spontaneous activity was recorded from BA1 under control conditions. This appeared on the recordings as rapid membrane fluctuations with amplitudes of 0.5 -1 mV which can be seen in Figure 5.5A and is caused by neurons which make synaptic contact with BA1. This spontaneous activity was inhibited within 1 minute following the application of DL-octopamine. The spontaneous activity returned, within 15 minutes after washing the preparation with drug-free saline. This indicates that DL-octopamine inhibits neurons presynaptic to BA1.

To summarise, the reversible decrease in the amplitude of the ACh responses recorded from BA1 following the application of DL-octopamine ( $10^{-5}$ - $10^{-4}$  M) shows that octopamine can modulate the cholinergic response of BA1. Therefore, octopamine is potentially capable of modulating synaptic transmission between the fSR and BA1 by inhibiting the response of BA1 to ACh released from the fSR terminals.

### 5.3.3. Effects of octopamine on ACh responses in the presence of scopolamine

The response of BA1 to ACh is elicited through the activation of cholinergic receptors. Previous work has shown that the application of the nicotinic cholinergic antagonists, gallamine and  $\alpha$ -bungarotoxin to this preparation substantially depresses the amplitudes of both electrically-evoked EPSPs and ACh responses recorded from BA1 (Leitch and Pitman, 1995). This indicates that nicotinic cholinergic receptors are mainly responsible for the response of BA1 to ACh. However, the cholinergic response was not completely inhibited in the presence of the nicotinic antagonists, indicating that other cholinergic receptors may play a role in evoking the cholinergic response in BA1. The results reported in Chapter 4 indicate that muscarinic cholinergic receptors on BA1 could also contribute to the cholinergic response. The application of muscarinic antagonists caused a slight decrease in the amplitude of the ACh response amplitude. Therefore, octopamine could potentially modulate the cholinergic response of BA1 by interfering with nicotinic and/or muscarinic cholinergic receptors. To investigate this, the muscarinic cholinergic receptors on BA1 were blocked with the muscarinic antagonist scopolamine and the effect octopamine had on the nicotinic cholinergic response of BA1 was examined.

To study the modulatory effects of octopamine on the nicotinic cholinergic response, the effects of octopamine on the response of BA1 to locally-applied ACh were examined, rather than the response of BA1 to the electrical stimulation of the fSR. This was to avoid the additional complexity of scopolamine blocking presynaptic muscarinic receptors, which would cause the amplitude of electrically-evoked EPSPs to increase

(see Chapter 4) and to avoid octopamine activating potential presynaptic octopamine receptors.

Under control conditions the application of brief pulses of ACh to the cell body of BA1 resulted in depolarising responses with an amplitude of  $20.75 \pm 3.6$  mV (mean  $\pm$  SEM;  $n = 3$ ). The muscarinic antagonist, scopolamine, was applied at a concentration of  $10^{-6}$  M. This concentration had been shown in Chapter 4 to be low enough to selectively block muscarinic receptors without blocking postsynaptic nicotinic receptors.

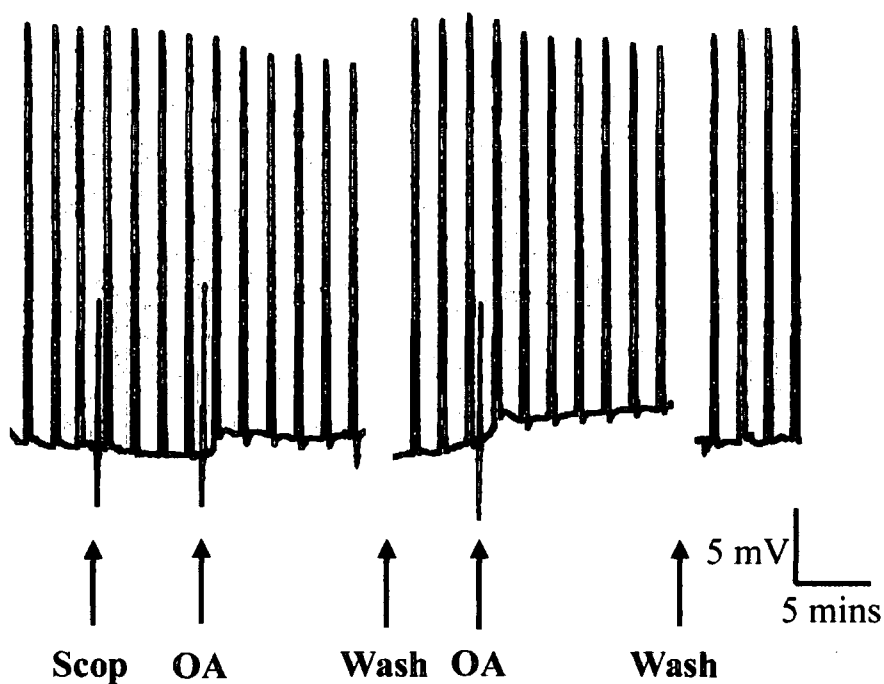
Application of scopolamine ( $10^{-6}$  M) resulted in a slight decrease in the ACh response amplitude of 2.2 % (+SEM 1.1; -SEM 1.5;  $p = 0.1917$ ;  $n = 3$ ). Scopolamine ( $10^{-6}$  M) did not cause a change in the membrane potential of BA1 ( $n = 3$ ).

DL-Octopamine ( $10^{-4}$  M) was then bath applied in the presence of scopolamine (Figure 5.6A). The amplitude of the nicotinic ACh responses began to decrease within 1 minute following the bath application of DL-octopamine in the presence of scopolamine and had reached a maximum decrease within 10 minutes. The amplitude of the nicotinic ACh responses recorded from BA1 decreased by a mean percentage of 18.6 % (+SEM 4.4; -SEM 4.9;  $p = 0.0067$ ;  $n = 3$ ) (Figure 5.6B). The membrane potential of BA1 initially depolarised by 1.5 -5 mV following DL-octopamine application but did begin to repolarise within 10 minutes. After washing the preparation with drug-free saline the amplitude of the ACh responses recorded from BA1 returned to approximately control levels.

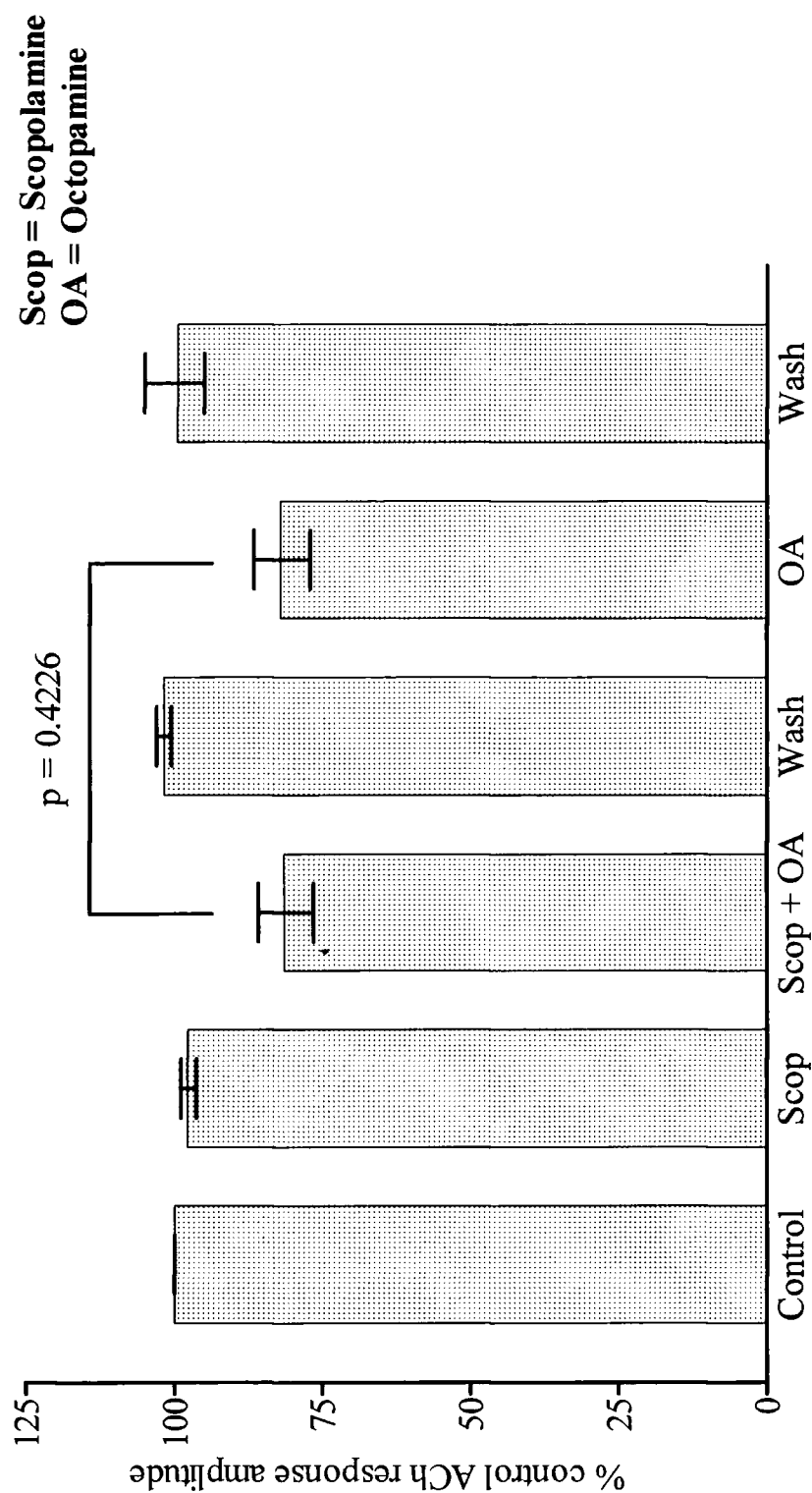
After the preparation had been washed thoroughly and a set of ACh responses had been recorded with amplitudes similar to control amplitudes, DL-octopamine ( $10^{-4}$  M) was

**Figure 5.6A. Effects of Octopamine ( $10^{-4}$  M) on ACh responses recorded from BA1 in the presence of Scopolamine ( $10^{-6}$  M).** Brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of scopolamine ( $10^{-6}$  M) caused a slight decrease in the amplitude of the ACh response. Application of octopamine ( $10^{-4}$  M) in the presence of scopolamine caused a decrease in the ACh response amplitude which reversed after washing. Bath application of octopamine ( $10^{-4}$  M) alone to the same preparation, caused a decrease in the ACh response amplitude that was similar to that caused by octopamine in the presence of scopolamine.

**Figure 5.6A. Effects of Octopamine ( $10^{-4}$  M) on ACh responses recorded from BA1 in the presence of Scopolamine ( $10^{-6}$  M).**







**Figure 5.6B. Effects of Octopamine ( $10^{-4}$  M) on ACh response amplitude in the presence of Scopolamine ( $10^{-6}$  M).** Graphical representation of pooled arc sine transformed data showing the mean ( $\pm$  SEM) amplitude of ACh responses ( $n=3$ ). The amplitude is given as a percentage of the control amplitude. The ACh response amplitude following octopamine application is not significantly different in the presence or absence of scopolamine in the same preparation. The P value was calculated using a two-tailed t-test.

bath applied alone to the same preparation. This resulted in the amplitude of the ACh responses recorded from BA1 decreasing by 17.9 % (+SEM 4.5; -SEM 5.0;  $p = 0.0496$ ;  $n = 3$ ). This reversed after washing the preparation with drug-free saline.

Therefore, the percentage decrease in the ACh response amplitude following the bath application of DL-octopamine was not significantly different ( $p = 0.4226$ ) in either the absence or presence of the muscarinic antagonist, scopolamine ( $10^{-6}$  M). This indicates that octopamine modulates the cholinergic response of BA1 by interfering with nicotinic rather than muscarinic cholinergic receptors.

To summarise, the decrease in the electrically-evoked EPSP amplitude and duration following the application of DL-octopamine indicates that octopamine is potentially capable of inhibiting cholinergic synaptic transmission between the fSR and BA1. The decrease in the amplitude of ACh responses following the application of DL-octopamine suggests octopamine inhibits synaptic transmission at the fSR/ BA1 synapse (at least in part) by inhibiting the response of BA1 to ACh. The effects of octopamine on the cholinergic response of BA1 do not appear to be due to changes in the membrane conductance of BA1 and the present set of results indicates that the cholinergic response of BA1 is modulated by octopamine interfering with nicotinic rather than muscarinic cholinergic receptors.

#### **5.3.4. Characterisation of receptors mediating the effects of octopamine**

To characterise the receptors on BA1 that may be activated by octopamine, the effects of a variety of vertebrate aminergic agonists and antagonists were examined. Most of

these agents act at the vertebrate  $\alpha$ -adrenergic receptor but are also reported to act at invertebrate octopamine receptors (Nathanson and Greengard, 1973; Evans, 1981; Orr and Hollingworth, 1990; Saudou et al., 1990; Orr et al., 1991; Roeder, 1992, 1995; Hiripi et al., 1998). The vertebrate  $\alpha$ -adrenergic receptor antagonist, tolazoline and the vertebrate  $\alpha$ -adrenergic receptor agonist, naphazoline are reported to act as insect octopamine receptor agonists (Evans, 1981; Roeder, 1992, 1995), whereas the vertebrate  $\alpha$ -adrenergic receptor antagonists, phentolamine and mianserin and the vertebrate dopamine antagonist, metoclopramide are reported to act as invertebrate octopamine receptor antagonists (Nathanson and Greengard, 1973; Evans, 1981; Orr et al., 1991; Roeder, 1992; Hiripi et al., 1998). The rank order of the affinities of these agonists and antagonists for octopamine receptors has been used to characterise insect octopamine receptor classes (see Table 5.1).

#### **5.3.4.1. Effects of insect octopamine receptor agonists on ACh responses**

Application of the agonists, tolazoline and naphazoline, mimicked the action of DL-octopamine by causing a decrease in the amplitude of ACh responses recorded from BA1 ( $n = 4$ ). Tolazoline ( $10^{-4}$  M) caused the ACh response amplitude to begin to decrease within 3 minutes of bath application and within 10 minutes it had reached its maximal effect (Figure 5.7A). Tolazoline ( $10^{-4}$  M) caused a mean decrease of 18.5% ( $\pm$  SEM 0.5;  $p = 0.0092$ ) in the ACh response amplitude. This decrease in ACh response amplitude was not accompanied by a change in the input resistance of BA1 but in one preparation the membrane potential of BA1 hyperpolarised by 3 mV and, in another, the membrane potential depolarised by 1.5 mV, following tolazoline application. However, following current injection to return BA1's membrane potential to control

levels, the ACh response amplitude was still decreased. This indicates that the decrease in ACh amplitude following tolazoline application was not due to changes in the membrane potential of BA1. After washing the preparation with drug-free saline the ACh response amplitude returned to control levels.

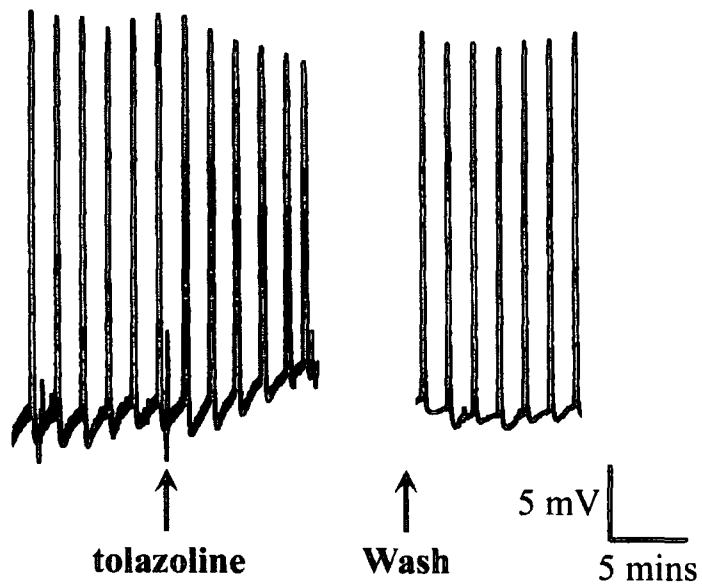
Following the bath application of naphazoline ( $10^{-4}$  M) the ACh response amplitude decreased markedly by a mean percentage of 67.5 % ( $\pm$  SEM 2.5;  $p = 0.0098$ ) (Figure 5.7B). This decrease in ACh response amplitude following naphazoline application was not accompanied by a change in the input resistance or membrane potential of BA1. The ACh response amplitude returned to control levels after more than 20 minutes of washing the preparation with drug-free saline.

A comparison of the overall percentage decreases in ACh response amplitude following the application of DL-octopamine, tolazoline and naphazoline at  $10^{-4}$  M, is shown in Figure 5.8. It demonstrates that DL-octopamine ( $10^{-4}$  M) caused a decrease in the ACh response amplitude that was not significantly greater ( $p = 0.6268$ ) than the amplitude decrease caused by tolazoline ( $10^{-4}$  M). Naphazoline ( $10^{-4}$  M), however, caused a markedly greater decrease in the ACh response amplitude recorded from BA1. The amplitude decrease caused by naphazoline ( $10^{-4}$  M) was significantly greater than the amplitude decrease caused by either DL-octopamine ( $10^{-4}$  M) ( $p = 0.0021$ ) or tolazoline ( $10^{-4}$  M) ( $p = 0.0028$ ). The rank order of effectiveness of these agonists on ACh response amplitudes correlates most closely with the rank order of affinities of these agonists for the insect octopamine<sub>2A</sub> receptor class (Table 5.1).

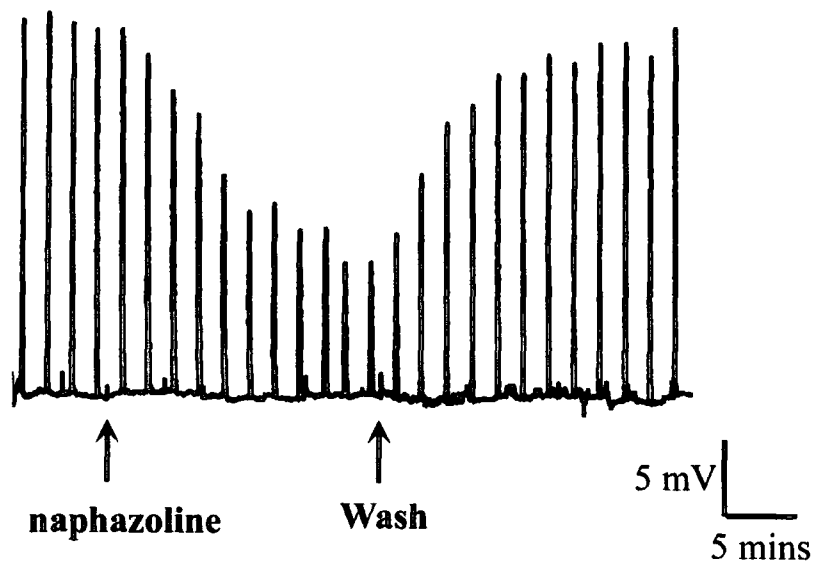
**Figure 5.7A. Effects of Tolazoline ( $10^{-4}$  M) on ACh responses recorded from BA1.** Brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of tolazoline ( $10^{-4}$  M) caused a decrease in the amplitude of the ACh response. The decrease in ACh response amplitude reversed after washing.

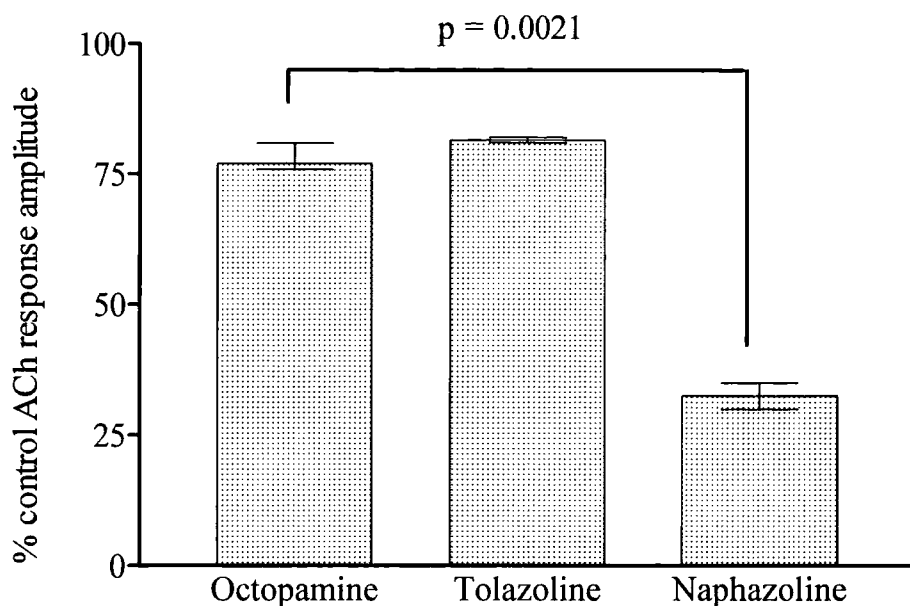
**Figure 5.7B. Effects of Naphazoline ( $10^{-4}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, resulted in depolarising responses in BA1. Bath application of naphazoline ( $10^{-4}$  M) caused a marked decrease in the amplitude of the ACh response. Washing with drug-free saline resulted in the ACh response amplitude returning to the control levels.

**Figure 5.7A. Effects of Tolazoline ( $10^{-4}$  M) on ACh responses recorded from BA1.**



**Figure 5.7B. Effects of Naphazoline ( $10^{-4}$  M) on ACh responses recorded from BA1.**





**Figure 5.8. Comparison of the percentage decreases in ACh response amplitude caused by insect octopamine receptor agonists.** Graphical representation of pooled data showing the mean ( $\pm$  SEM) ACh response amplitudes as a percentage of the control amplitude. Application of DL-octopamine ( $10^{-4}$  M) or tolazoline ( $10^{-4}$  M) resulted in a similar decrease in ACh response amplitude ( $p = 0.6268$ ) but naphazoline ( $10^{-4}$  M) caused a significantly greater decrease. The P values were calculated using a two-tailed t-test.

It is possible, however, (if unlikely) that tolazoline and naphazoline are actually modulating the cholinergic response of BA1 via a receptor class different to that upon which octopamine acts. To characterise the receptor upon which octopamine acts, the antagonism of the action of octopamine has to be examined. As with the agonists reported above, the rank order of the effectiveness of the antagonists, phentolamine, metoclopramide and mianserin can be used to characterise insect octopamine receptor classes (see Table 5.1). The effectiveness of these antagonists at blocking the inhibitory action of DL-octopamine, was therefore examined.

#### **5.3.4.2. Effects of insect octopamine receptor antagonists on ACh responses**

The results from the experiments revealed that all of the insect octopamine receptor antagonists tested, namely, phentolamine, mianserin and metoclopramide, had a purely agonistic rather than antagonistic action on ACh responses. Bath application of  $10^{-4}$ - $10^{-6}$  M phentolamine ( $n = 7$ ),  $10^{-4}$ - $10^{-5}$  M metoclopramide ( $n = 2$ ) and  $10^{-4}$  M mianserin ( $n = 2$ ) mimicked the effects of DL-octopamine and caused a decrease in the ACh response amplitude recorded from BA1 (Figures 5.9,10).

Bath application of phentolamine ( $10^{-4}$  M) caused a mean decrease of 82.6% (+ SEM 6.4; - SEM 7.6;  $p = 0.0517$ ) in the ACh response amplitude (Figure 5.9A). The ACh response partially reversed after washing the preparation with drug-free saline, to 63% (+ SEM 17.8; - SEM 16.2) of the control ACh response amplitude. Bath application of metoclopramide ( $10^{-4}$  M) caused a 68% decrease in the ACh response amplitude (Figure 5.9B). The decrease in the ACh response amplitude reversed to control amplitude levels after washing the preparation with drug-free saline. Mianserin also

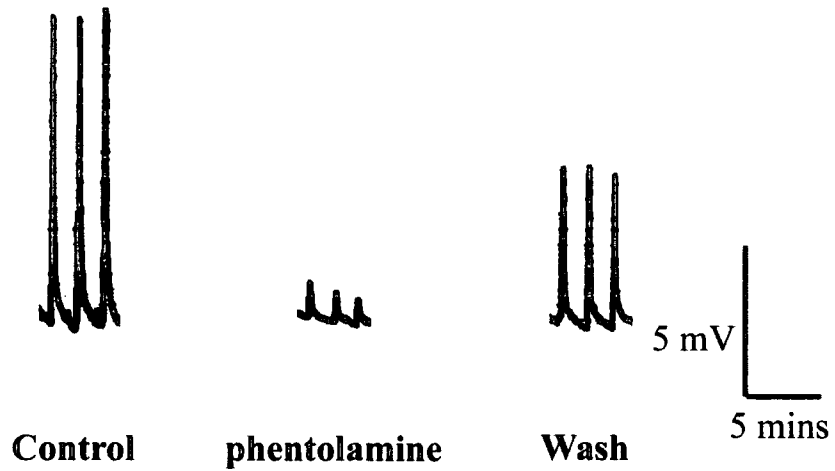


**Figure 5.9A. Effects of Phentolamine ( $10^{-4}$  M) on ACh responses recorded from BA1.** Brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of phentolamine ( $10^{-4}$  M) almost inhibited the ACh response completely. The ACh response returned partially, after washing.

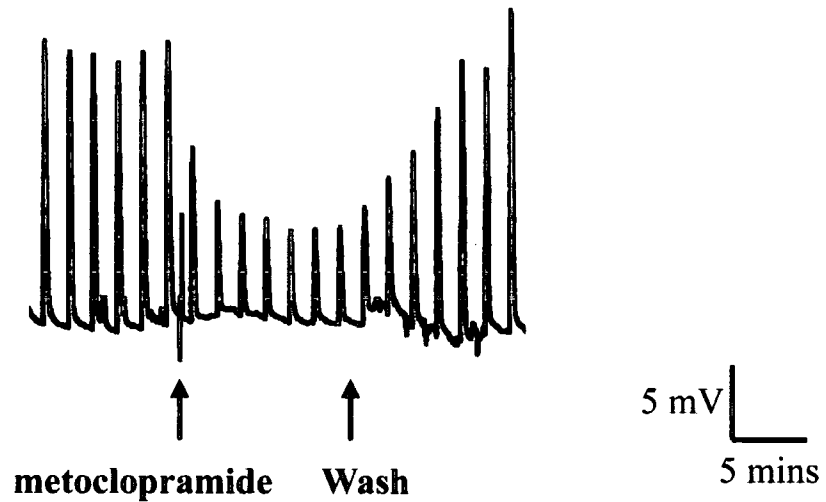
**Figure 5.9B. Effects of Metoclopramide ( $10^{-4}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, resulted in depolarising responses in BA1. Bath application of metoclopramide ( $10^{-4}$  M) caused a marked decrease in the amplitude of the ACh response. Washing with drug-free saline resulted in the ACh response amplitude returning to the control levels.

**Figure 5.9C. Effects of Mianserin ( $10^{-4}$  M) on ACh responses recorded from BA1.** Brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of mianserin ( $10^{-4}$  M) caused a marked decrease in the amplitude of the ACh response. The decrease in ACh response amplitude reversed partially after washing.

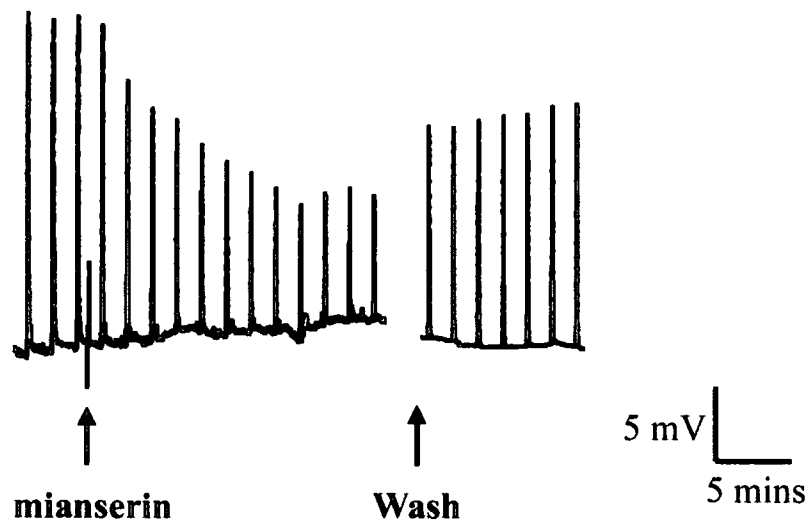
**Figure 5.9A. Effects of Phentolamine ( $10^{-4}$  M) on ACh responses recorded from BA1.**

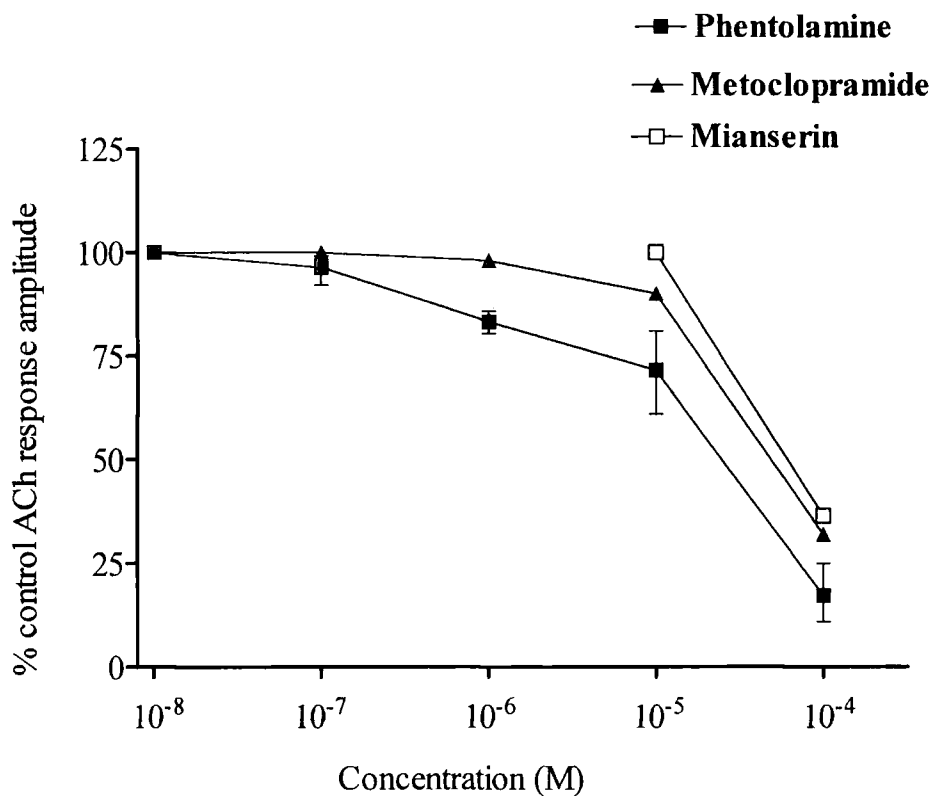


**Figure 5.9B. Effects of Metoclopramide ( $10^{-4}$  M) on ACh responses recorded from BA1.**



**Figure 5.9C. Effects of Mianserin ( $10^{-4}$  M) on ACh responses recorded from BA1.**





**Figure 5.10. Comparison of the concentration dependent agonistic effects of insect octopamine receptor antagonists on ACh response amplitudes.** Graphical representation of the pooled data showing the mean ( $\pm$  SEM) amplitude of ACh responses in the presence of different concentrations of either phentolamine, metoclopramide or mianserin. The amplitude is given as a percentage of the control amplitude.

caused a decrease in the ACh response amplitude recorded from BA1. Bath application of mianserin ( $10^{-4}$  M) caused the ACh response amplitude to decrease by 63.5% ( $\pm$  SEM 0.5;  $p = 0.0036$ ) (Figure 5.9C). After washing with drug-free saline, the ACh response amplitude partially reversed from 36.5% to 63.8% of the control ACh response amplitude.

Application of the antagonists at lower concentrations revealed that the agonistic action of the antagonists on ACh responses was concentration dependent (Figure 5.10). At  $10^{-5}$  M, phentolamine caused a reversible 28.5% (+ SEM 10.5; - SEM 9.5;  $p = 0.1249$ ) decrease and metoclopramide caused a reversible 10% decrease in the ACh response amplitude. Application of  $10^{-5}$  M mianserin on the other hand had no significant effect on the ACh response amplitude. Application of  $10^{-6}$  M phentolamine caused a 16.8% (+ SEM 2.8; - SEM 2.6;  $p = 0.0072$ ) decrease in the ACh response amplitude which was reversible after washing the preparation with drug-free saline. Application of  $10^{-6}$  M metoclopramide ( $n = 2$ ) had no significant effect on the ACh response amplitude though. Phentolamine at  $10^{-7}$  -  $10^{-8}$  M ( $n = 5$ ) and metoclopramide at  $10^{-7}$  -  $10^{-8}$  M ( $n = 2$ ) had no significant effect on the ACh response amplitude.

Comparison of the agonistic threshold concentration values of the insect octopamine receptor antagonists, and their concentration dependent agonistic effects on ACh response amplitudes (Figure 5.10), reveals a rank order of these compounds according to their agonistic effects. Phentolamine has the greatest agonistic effect causing the largest decrease in the ACh response amplitude at  $10^{-4}$  M and having the lowest threshold concentration ( $10^{-6}$  M). Metoclopramide has the second greatest agonistic effect causing the second largest decrease in the ACh response amplitude at  $10^{-4}$  M and

having the second lowest threshold concentration ( $10^{-5}$  M). Mianserin causes the smallest decrease in the ACh response amplitude at  $10^{-4}$  M and has the highest threshold concentration ( $10^{-4}$  M). This rank order of agonistic effectiveness however, cannot be used to characterise insect octopamine receptors.

In other studies (Evans, 1981; Kaufmann and Benson, 1991; Roeder, 1992) these compounds do antagonise octopamine. In the locust extensor-tibiae-neuromuscular preparation the responses to octopamine are blocked by phentolamine, mianserin and metoclopramide ( $10^{-6}$ - $10^{-5}$  M) and the responses of isolated locust central neurons to octopamine are blocked by phentolamine ( $10^{-6}$ - $10^{-4}$  M) and mianserin ( $10^{-9}$ - $10^{-7}$  M) (Kaufmann and Benson, 1991). In addition, binding studies have shown that these agents ( $10^{-6}$ - $10^{-2}$  M) can displace  $^3\text{H}$ -octopamine binding in the locust CNS (Roeder, 1992). The rank order of the antagonistic effectiveness of these agents has been used to characterise octopamine receptor classes (Evans, 1981; Roeder, 1992). Therefore, the antagonistic effects of these compounds on the octopaminergic modulation of ACh responses recorded from BA1, were examined by applying the antagonists at concentrations shown by the above experiments not to affect the amplitude of the ACh response recorded from BA1. DL-Octopamine ( $10^{-4}$  M) was then applied in the presence of the antagonists. In most preparations, DL-octopamine ( $10^{-4}$  M) was also applied in the absence of the antagonists by thoroughly washing the preparation of both the antagonist and DL-octopamine and then reapplying octopamine alone.

#### **5.3.4.3. Effects of octopamine on ACh responses in the presence of insect octopamine receptor antagonists**

The results from the experiments revealed that none of the insect octopamine receptor antagonists tested, antagonised the octopaminergic modulation of ACh responses recorded from BA1.

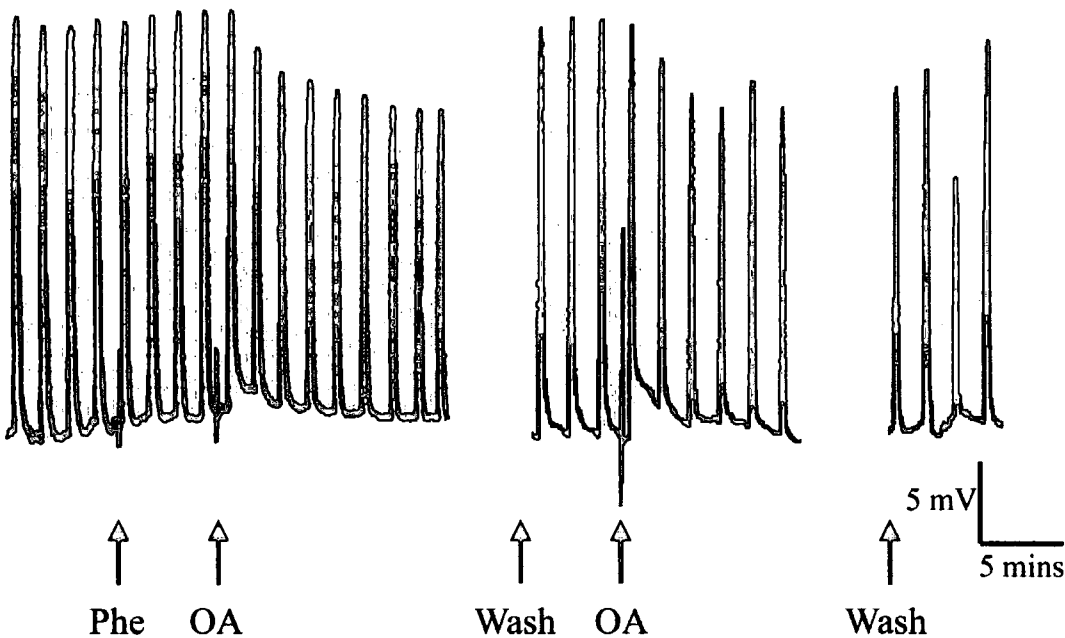
Bath application of DL-octopamine ( $10^{-4}$  M), in the presence of phentolamine ( $10^{-8}$  M), caused a 22.9% ( $\pm$  SEM 1;  $p = 0.0053$ ;  $n = 2$ ) decrease in the ACh response amplitude (Figure 5.11A). After the preparation had been washed thoroughly and a set of ACh responses had been recorded with amplitudes similar to control levels, DL-octopamine ( $10^{-4}$  M) was applied alone to the same preparation. DL-Octopamine ( $10^{-4}$  M) applied alone caused a 20.9% (+ SEM 2.9; - SEM 3.1;  $p = 0.0057$ ;  $n = 2$ ) decrease in the ACh response amplitude. Therefore, the percentage decrease in the ACh response amplitude following DL-octopamine application was not significantly different ( $p = 0.5$ ) in the presence or absence of phentolamine ( $10^{-8}$  M). This indicates that phentolamine at this particular concentration, does not antagonise the octopaminergic modulation of ACh responses recorded from BA1.

Similar effects were found with metoclopramide ( $10^{-6}$  M). DL-Octopamine ( $10^{-4}$  M) caused a 12.9 % (+ SEM 1.9; - SEM 2.1;  $p = 0.0104$ ;  $n = 2$ ) decrease in the ACh response amplitude in the presence of metoclopramide ( $10^{-6}$  M) and a decrease of 12.5 % when applied alone to the same preparation (Figure 5.11B). Therefore, metoclopramide, at this particular concentration, does not appear to antagonise the action of octopamine on the ACh responses.

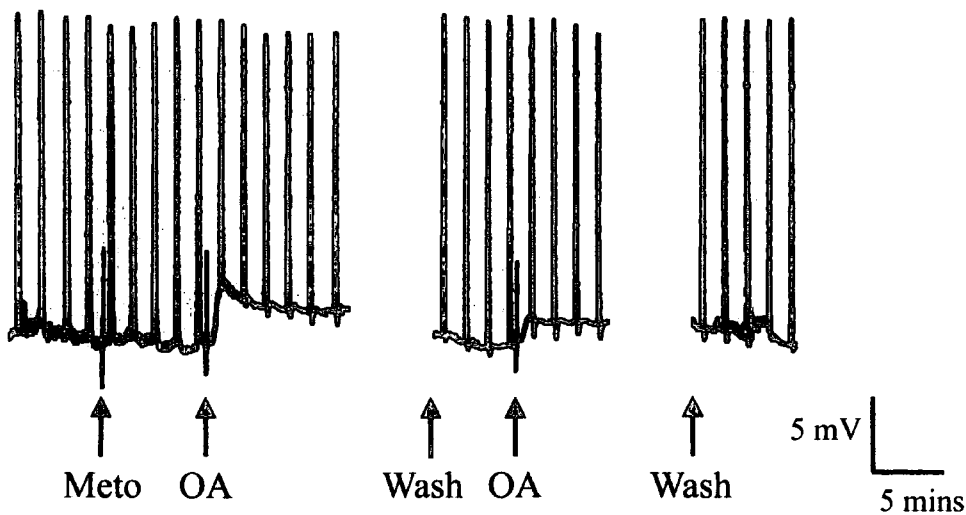
**Figure 5.11A. Effect of Octopamine ( $10^{-4}$  M) on ACh responses in the presence of Phentolamine ( $10^{-8}$  M).** Brief pulses of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of phentolamine ( $10^{-8}$  M) had no effect on the amplitude of the ACh response. Bath application of octopamine ( $10^{-4}$  M) in the presence of phentolamine, caused a decrease in the ACh response amplitude which was similar to the decrease when octopamine ( $10^{-4}$  M) was applied alone, to the same preparation. Phe = phentolamine, OA = octopamine.

**Figure 5.11B. Effect of Octopamine ( $10^{-4}$  M) on ACh responses in the presence of Metoclopramide ( $10^{-6}$  M).** Pressure-application of brief pulses of acetylcholine (ACh) to the cell body of BA1, caused depolarising responses in BA1. Application of metoclopramide ( $10^{-6}$  M) had no effect on the amplitude of the ACh response. Bath application of octopamine ( $10^{-4}$  M) in the presence of metoclopramide caused a decrease in the ACh response amplitude that was reversible with washing. Application of octopamine ( $10^{-4}$  M) alone to the same preparation, caused a decrease in the ACh response amplitude that was similar to the decrease when octopamine was applied in the presence of metoclopramide. Meto = metoclopramide, OA = octopamine.

**Figure 5.11A. Effect of Octopamine ( $10^{-4}$  M) on ACh responses in the presence of Phentolamine ( $10^{-8}$  M).**



**Figure 5.11B. Effect of Octopamine ( $10^{-4}$  M) on ACh responses in the presence of Metoclopramide ( $10^{-6}$  M).**





To summarise, application of the insect octopamine receptor antagonists at concentrations that had no agonistic effect did not antagonise the octopaminergic modulation of ACh responses. It is possible, however, that at higher concentrations the insect octopamine receptor antagonists may antagonise the action of octopamine on the ACh responses. This would be difficult to determine though, due to the fact that these particular antagonists produced agonistic effects on the ACh responses in our preparation. Therefore, the results from these experiments, which examined the effects of insect octopamine receptor antagonists on ACh responses, cannot be used to characterise the receptors involved in the octopamine-mediated modulation of ACh responses recorded from BA1.

#### **5.3.5. Effect of insect octopamine receptor antagonists on electrically-evoked EPSPs**

The results presented so far demonstrate that cholinergic synaptic transmission between the fSR and BA1 is modulated by octopamine and indicate that the modulation is mediated (at least in part) via receptors located on the postsynaptic neuron, BA1. The results from the experiments which examined the effects of insect octopamine receptor agonists on ACh responses suggest that the modulation may be mediated via octopamine<sub>2A</sub> receptors on BA1. This cannot be verified, however, by the results from the experiments which examined the effects of insect octopamine receptor antagonists on ACh responses. This was because the antagonists had a purely agonistic rather than antagonistic action on ACh responses i.e. they mimicked the inhibitory effects of DL-octopamine and caused a decrease in the ACh response amplitudes recorded from BA1.

Surprisingly the results, from experiments which examined the effects of the insect octopamine receptor antagonists on electrically-evoked EPSPs, revealed that the vertebrate  $\alpha$ -adrenergic antagonists, phentolamine and mianserin, caused a reversible increase in the electrically-evoked EPSP amplitude.

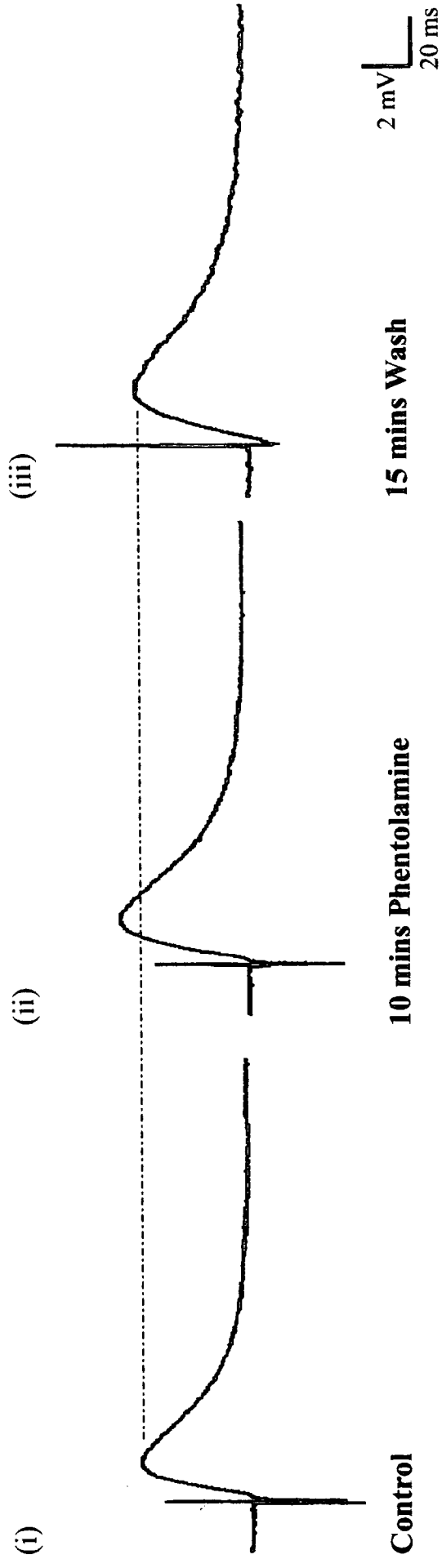
Bath application of phentolamine ( $10^{-4}$  M) caused an increase of 16.1% (+ SEM 6.5; - SEM 7.2;  $p = 0.0075$ ;  $n = 5$ ) in the EPSP amplitude (Figure 5.12). The increase in EPSP amplitude following phentolamine application was not accompanied by a change in the membrane potential or input resistance of BA1. The EPSP amplitude returned to control levels 15 minutes after washing the preparation with drug-free saline (Figure 5.12).

Similarly, bath application of mianserin ( $10^{-4}$  M) caused a 21.2% (+ SEM 6.5; - SEM 6.1;  $p = 0.0319$ ;  $n = 5$ ) increase in the EPSP amplitude (Figure 5.13). The EPSP amplitude began to increase within 5 minutes following application and had generally reached a maximum amplitude within 30 minutes. The increase in EPSP amplitude following mianserin application was not accompanied by a change in the membrane potential or the input resistance of BA1. The increase in EPSP amplitude reversed to control levels 15 minutes after washing the preparation with drug-free saline (Figure 5.13).

The effect of the vertebrate dopamine antagonist, metoclopramide, on the amplitude of electrically-evoked EPSPs was, however, similar to the effect it had on the amplitude of ACh responses. Bath application of metoclopramide caused a decrease in the

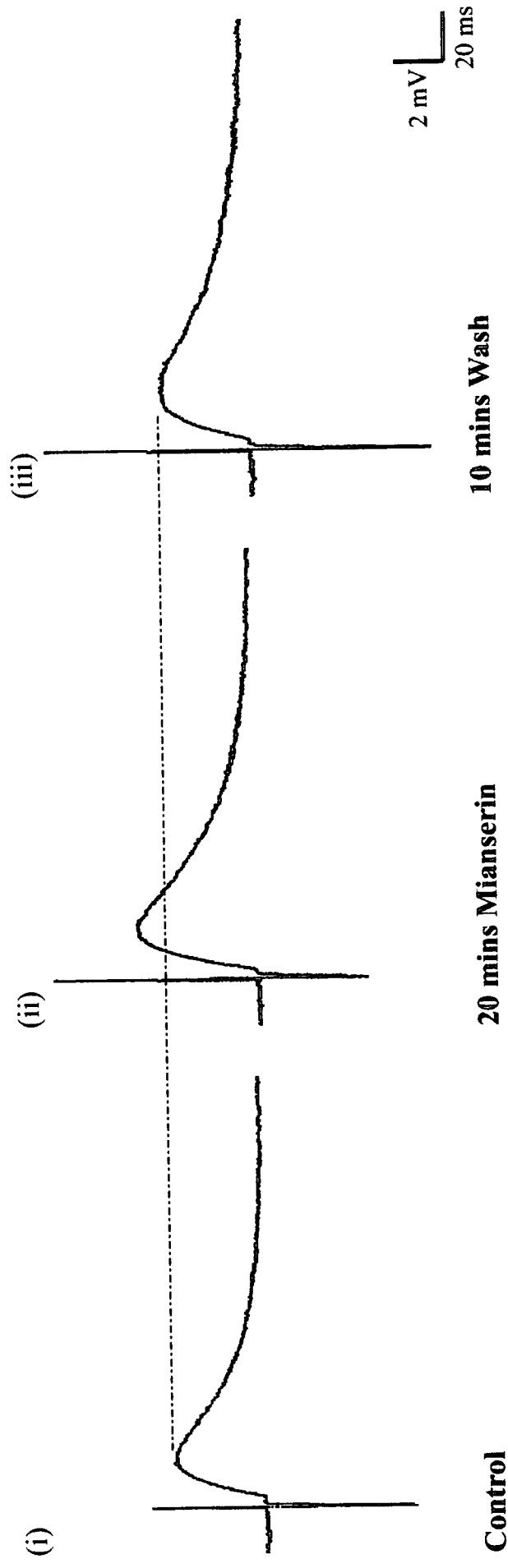
**Figure 5.12. Effect of Phentolamine ( $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) Following the application of phentolamine ( $10^{-4}$  M), the EPSP amplitude increased. (iii) The increase in EPSP amplitude returned to control levels after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

**Figure 5.12. Effect of Phentolamine ( $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1.**



**Figure 5.13. Effect of Mianserin ( $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) Following the application of mianserin ( $10^{-4}$  M), the EPSP amplitude increased. (iii) The increase in EPSP amplitude returned to control levels after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.

**Figure 5.13. Effect of Mianserin ( $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1.**



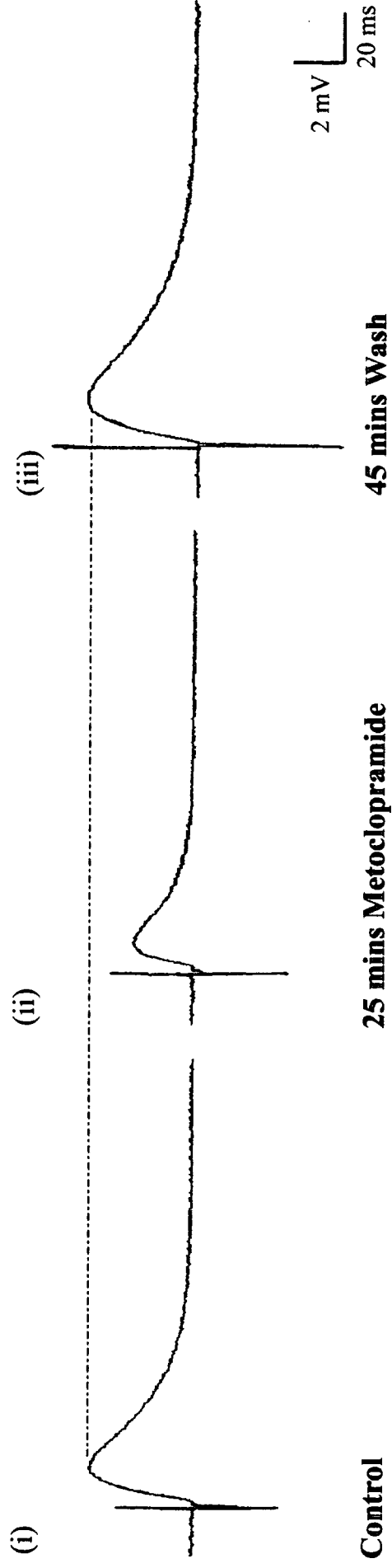
electrically-evoked EPSP amplitude ( $n = 6$ ). Application of metoclopramide ( $10^{-4}$  M) caused a 64.4% (+ SEM 1.1; - SEM 1;  $p = 0.0284$ ) decrease in the amplitude of the electrically-evoked EPSP. The EPSP amplitude began to decrease within 5 minutes following metoclopramide application and had generally reached a maximal effect within 30 minutes. The decrease in EPSP amplitude was not accompanied by a change in the membrane potential or input resistance of BA1. Application of lower concentrations of metoclopramide revealed the effect of metoclopramide on the amplitude of the EPSP was concentration dependent. Bath application of metoclopramide ( $10^{-5}$  M) caused a 45.8% ( $\pm$  SEM 0.7;  $p = 0.01$ ) decrease in the amplitude of the electrically-evoked EPSP (Figure 5.14) whereas metoclopramide ( $10^{-6}$  M) had no effect on the EPSP amplitude. Therefore, the threshold concentration at which metoclopramide had an effect on the EPSP amplitude was  $10^{-5}$  M, the same as the threshold concentration on ACh responses.

Comparison of the effects of the  $\alpha$ -adrenergic antagonists, phentolamine ( $10^{-4}$  M) and mianserin ( $10^{-4}$  M) on electrically-evoked EPSPs and ACh responses reveal that, phentolamine and mianserin caused a 16.1% and 21.2% increase in the EPSP amplitude respectively, but a 82.6% and 63.5% decrease in the ACh response amplitude. The increase in the electrically-evoked EPSP amplitudes caused by phentolamine and mianserin is not due to their action on the postsynaptic neuron BA1, as the experiments described previously revealed that phentolamine and mianserin cause a decrease in the ACh response amplitudes. This indicates that the increase in the electrically-evoked EPSP amplitudes caused by phentolamine and mianserin, is due to the antagonists acting at receptors located presynaptically.

**Figure 5.14. Effect of Metoclopramide ( $10^{-5}$  M) on electrically-evoked EPSPs recorded from BA1.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) Metoclopramide ( $10^{-5}$  M) caused a decrease in the EPSP amplitude. (iii) This decrease was reversible after washing. Each trace is an average of 8 successive electrically-evoked EPSPs.



**Figure 5.14. Effect of Metoclopramide ( $10^{-5}$  M) on electrically-evoked EPSPs.**



### 5.3.6. Octopamine-immunoreactivity in aLAC

The results presented in this chapter clearly demonstrate that octopamine is potentially capable of modulating the fSR/BA1 synapse. This, however, does not confirm that octopamine modulates the fSR/BA1 synapse under normal physiological conditions. Octopamine would have to be at least present within the neuropil region that contains the fSR/BA1 synapses to be capable of modulating synaptic transmission between the fSR and BA1. The dendrites of the fSR and flight motoneurons project into aLAC in the dorso-lateral neuropil region of the mesothoracic ganglion. The presence of octopamine in aLAC would, therefore, indicate octopamine could at least potentially modulate the fSR/BA1 synapse. A previous study (Stevenson et al., 1992), detailing the distribution of OA-IR in the mesothoracic ganglion of the locusts *Locusta migratoria* and *Schistocerca gregaria*, noted the presence of OA-IR processes in the dorso-lateral neuropils but did not identify the specific neuropils expressing OA-IR. Therefore, this study aimed to establish if octopamine was present in aLAC, using confocal microscopical immunocytochemistry.

The distribution of OA-IR in the mesothoracic ganglion was first assessed, to confirm that the pattern of labelling in this study was consistent with that reported previously by Stevenson et al. (1992). Transverse sections through the mesothoracic ganglion which had been incubated with an anti-octopamine antibody and labelled with Rhodamine Red were examined under an inverted Nikon Axiophot microscope equipped with a Biorad Microradiance confocal system. Primary antibody omission controls were also inspected. Examination of the slices which had been incubated with the anti-octopamine antibody revealed specific regions of red fluorescence which indicated OA-

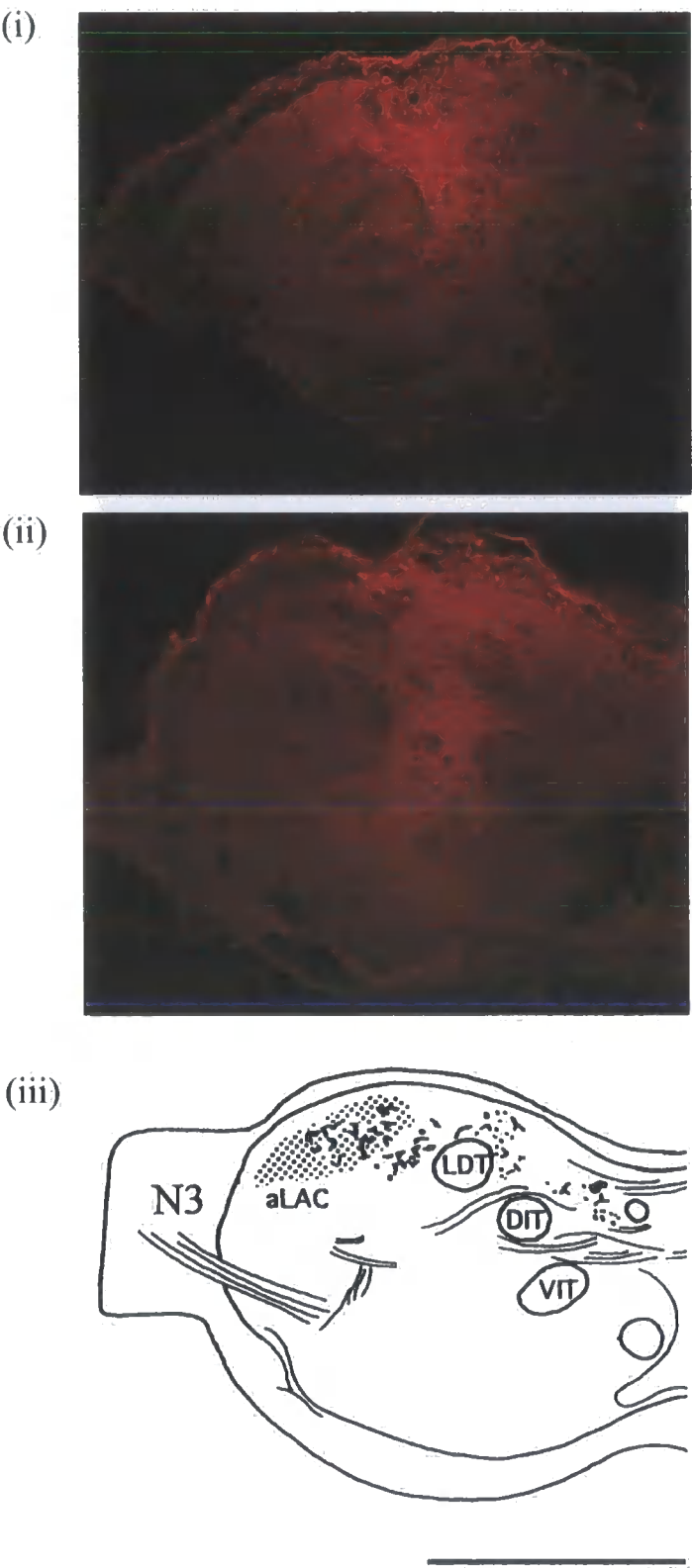
IR. Primary antibody omission controls showed no red fluorescence. The level at which each slice had been taken through the mesothoracic ganglion was identified under light microscopical conditions using anatomical guides (Tyrer and Gregory, 1982; Pflüger et al., 1988). Therefore, the transverse sections which contained aLAC could be identified. Camera lucida drawings of the levels through the mesothoracic ganglion containing this neuropil region, are shown in Chapter 4.

The transverse slices taken at the level of the emergence of N3 contain this neuropil region. Identified transverse sections taken at this level are shown in Figure 5.15A. Examination of these slices revealed that the OA-IR represented by red fluorescence, was most intense in the dorsal region of each slice, which coincides with the aLAC neuropil region. This concentration of OA-IR extended from the dorsal edge of the mesothoracic ganglion ventrally to approximately the level of the ventral intermediate tract (VIT) and from the midline laterally towards the roots of N3. OA-IR was most intense within this region, medial to the LDT and lateral to the DIT (Figure 5.15Ai). Individual processes were seen most clearly within this region, close to the dorsal edge in the area of the medial dorsal tract (MDT) (Figure 5.15Aii). OA-IR was also present in the ventral lateral neuropil regions of the ganglia, although the fluorescence was not as intense. Cell bodies on the surface of the ganglia did not appear to show any OA-IR. Comparison of the confocal images showing the OA-IR distribution and the camera lucida drawing showing the distribution of the fSR branches in Figure 5.15A, clearly demonstrates that the branches of the fSR and aLAC are in an area which is OA-IR.

The transverse slices taken at levels posterior to N3 do not contain aLAC but were examined to assess the specificity of the antibody labelling. Slices taken from the level

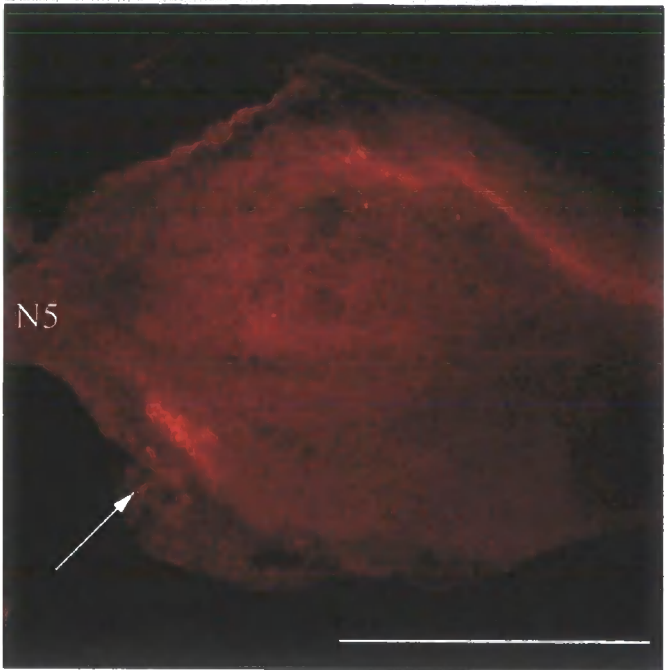
**Figure 5.15A. Octopamine-immunoreactivity in the mesothoracic ganglion. Level 1.** Transverse sections taken through the mesothoracic ganglion at the level of nerve 3 (N3). (i, ii) Confocal microscope images of transverse slices incubated with an anti-octopamine antibody and labelled with Rhodamine Red. Octopamine-immunoreactivity is indicated by the red fluorescence and is most dense in the dorsal region of the mesothoracic ganglion. (iii) Camera lucida drawing showing the distribution of the fSR branches and the position of the anterior lateral association centre (aLAC) (stippled area) in the dorsal region of mesothoracic ganglion. Dorsal is to the top, lateral is to the right. DIT = dorsal intermediate tract, LDT = lateral intermediate tract, VIT = ventral intermediate tract. Scale bar = 200  $\mu\text{m}$ .

**Figure 5.15A. Octopamine-immunoreactivity in the mesothoracic ganglion. Level 1.**



**Figure 5.15B. Octopamine-immunoreactivity in the mesothoracic ganglion. Level 2.** Confocal microscope image of a transverse section taken through the mesothoracic ganglion at the level of nerve 5 (N5). The slice has been incubated with an anti-octopamine antibody and labelled with Rhodamine Red. Octopamine-immunoreactivity is indicated by the red fluorescence. Octopamine-immunoreactivity is most dense in the dorsal region of the mesothoracic ganglion and in a small region near to the ventral edge of the mesothoracic ganglion, close to the emergence of N5 (indicated by arrow) which is consistent with the pattern of labelling described by Stevenson et al. (1992). Dorsal is to the top, lateral is to the right. Scale bar = 200  $\mu\text{m}$ .

**Figure 5.15B. Octopamine-immunoreactivity in the mesothoracic ganglion. Level 2.**



nerve 5 (N5) starts to emerge revealed a similar pattern of OA-IR distribution to those taken at the level of N3 (Figure 5.15B). The most dense OA-IR was in the dorsal region of the mesothoracic ganglion. However, a small region of intense OA-IR was also evident in the ventral region of the mesothoracic ganglion. This was located close to the lateral ventral edge of the ganglion near to the emergence of N5.

The OA-IR distribution in the mesothoracic ganglion, revealed in this confocal microscopical immunocytochemical study, is consistent with the distribution of OA-IR reported previously (Stevenson et al., 1992), indicating the labelling is specific. Therefore, the intense red fluorescence revealed in the dorsal lateral neuropil region of the mesothoracic ganglion indicates that octopamine is present in the neuropil region where it could potentially modulate the fSR/BA1 synapse.

#### **5.4. DISCUSSION**

The results from electrophysiological experiments presented clearly demonstrate that cholinergic synaptic transmission between the sensory afferent, fSR and the motoneuron, BA1 is modulated by the monamine, octopamine. The results indicate that octopamine modulates synaptic transmission by suppressing the response of the postsynaptic neuron, BA1, to ACh by interfering with nicotinic rather than muscarinic cholinergic receptors. The receptors on BA1, which may mediate the octopaminergic modulation of the cholinergic response, could not be characterised though, as the insect octopamine receptor antagonists used to characterise the receptors had complex actions. There is evidence, however, which indicates that some of the antagonists act at receptors located presynaptically, that may be involved in the modulation of the



fSR/BA1 synapse. Complementary to the results from the electrophysiological experiments is the presence of octopamine-immunoreactivity in the neuropil region containing the fSR/BA1 synapses, which supports a role for octopamine in the modulation of synaptic transmission between the fSR and BA1. The behavioural significance of this octopaminergic modulation is discussed.

#### **5.4.1. Octopaminergic modulation of cholinergic transmission**

The results presented clearly demonstrate that cholinergic synaptic transmission between the sensory afferent fSR and the motoneuron, BA1, is modulated by octopamine. Octopaminergic modulation of cholinergic transmission between a sensory afferent and a motoneuron has not been reported previously. The only other study to report octopaminergic modulation of cholinergic synaptic transmission between identified neurons in an insect preparation was on interneurons involved in the escape response in the cockroach, *Periplaneta americana* (Casagrand and Ritzmann, 1992a,b). Synaptic transmission between the giant interneurons and thoracic interneurons involved in the cockroach escape response was shown to be cholinergic (Casagrand and Ritzmann, 1992b). A following study revealed that application of octopamine ( $10^{-4}$ - $10^{-2}$  M) caused an increase in the amplitude of synaptic potentials recorded from the thoracic interneurons (Casagrand and Ritzmann, 1992a). An increase in the amplitude of postsynaptic cholinergic responses following octopamine application has also been reported in a recent study on the mushroom bodies of the honey bee, *Apis mellifera* (Oleskevich, 1999). The amplitude of electrically-evoked postsynaptic responses recorded from the mushroom body calyx, which were shown to be mediated by ACh, increased by 129% in the presence of octopamine ( $10^{-4}$  M). These studies demonstrate

that octopamine can enhance cholinergic synaptic transmission at certain insect synapses. The results presented in this chapter show that octopamine inhibited cholinergic synaptic transmission, as the amplitude and duration of electrically-evoked EPSPs recorded from BA1, decreased in the presence of octopamine.

Octopamine may modulate cholinergic synaptic transmission presynaptically by regulating the release of ACh from the cholinergic terminals and/or postsynaptically by modulating the cholinergic response of the postsynaptic neuron. The effect of octopamine on the response of BA1 to locally-applied ACh revealed that octopamine modulated synaptic transmission between the fSR and BA1 by reducing the cholinergic response of BA1. Bath application of octopamine ( $10^{-4}$  M) caused a 23% decrease in the amplitude of the ACh responses recorded from BA1. Similar findings were reported in a study on the cholinergic responses of the  $D_f$  motoneuron which is involved in the escape pathway in the cockroach, *Periplaneta americana* (Butt and Pitman, 1998). Application of octopamine ( $10^{-4}$  M) caused a decrease of 25.8% in the amplitude of the ACh responses recorded from  $D_f$ . Interestingly, a number of other results from a further study on the effect of octopamine on the cholinergic responses of  $D_f$  (Butt and Pitman, personal communication) were also similar to those reported in this study. Firstly, spontaneous activity recorded from both the motoneurons,  $D_f$  and BA1, was reduced in the presence of octopamine ( $10^{-4}$  M) and returned after washing the preparations with drug-free saline. Secondly, the octopaminergic modulation of the cholinergic response from both  $D_f$  and BA1 was due to the modulation of the cholinergic response mediated by nicotinic rather than muscarinic cholinergic receptors. Postsynaptic nicotinic cholinergic receptors were also responsible for the cholinergic postsynaptic responses recorded from the mushroom body calyx which were reported to be modulated by

octopamine (Oleskevich, 1999). Therefore, results from experiments so far reveal that all postsynaptic cholinergic responses modulated by octopamine are mediated by postsynaptic nicotinic cholinergic receptors.

There is evidence that octopamine not only regulates cholinergic synaptic transmission by modulating the response of the postsynaptic neuron to ACh, but may regulate the release of ACh from the presynaptic terminals. Although there is no direct evidence that octopamine regulates ACh release, octopamine has been reported to modulate the responses of cholinergic neurons (Ramirez and Orchard, 1990), which would in turn potentially regulate ACh release. The responses of the locust sensory afferent fSR (which had been shown to be cholinergic [Leitch and Pitman, 1995] ) to wing movements increases following the application of octopamine (Ramirez and Orchard, 1990). The responses of a number of other insect sensory afferents which are thought to be cholinergic are also reported to be modulated by octopamine. Octopamine increases adaptation in the cockroach femoral tactile spine by elevating the resting threshold (Zhang et al., 1992), it increases the number and frequency of spikes in the FeCO position sensitive neurons in the stick insect (Ramirez et al., 1993) and the locust (Matheson, 1997) and it increases the response of most of the units in the locust hindleg strand receptors to mechanical stimulation (Bräunig and Eder, 1998). Afferents which relay olfactory information from the antennal lobe to the mushroom bodies in the honey bee are also modulated by octopamine (Oleskevich, 1999). The amplitude of the presynaptic afferent fiber volley evoked by the electrical stimulation of the antennal lobe decreased following the application of octopamine in some animals. Octopaminergic modulation of these cholinergic afferents allows the sensory information carried by these neurons from the periphery to be regulated before it is

integrated into the CNS. However, modulation of cholinergic neurons is not confined to insect sensory neurons. A cholinergic interneuron, SO in the pond snail *Lymnaea stagnalis*, is reported to be modulated by octopaminergic neurons involved in feeding (Vehovszky et al., 1998). Intracellular stimulation of the octopaminergic neurons for 20-30 seconds evoked bursts of action potentials in the cholinergic SO interneuron. Therefore, octopaminergic modulation of the SO interneuron may regulate ACh release onto the pretractor and retractor motoneurons upon which the SO acts. Another way octopamine may modulate cholinergic synaptic transmission is by regulating choline uptake. As choline uptake can limit the rate ACh is synthesised, octopaminergic modulation of choline uptake can potentially regulate ACh release from the presynaptic terminals. The high affinity choline uptake system in the horseshoe crab, *Limulus polyphemus*, has been reported to be modulated by octopamine (Ford et al., 1995). Application of octopamine ( $10^{-5}$  M) increased choline uptake by 19.4 %. This study and the others mentioned above provide evidence as to how cholinergic synaptic transmission between neurons in invertebrate preparations may be modulated by octopamine. Octopamine may; a) alter the response of the postsynaptic neuron to ACh, b) regulate ACh release from the presynaptic terminals or c) modulate choline uptake.

There is very little evidence reported so far, to suggest that octopamine is involved in modulating cholinergic synaptic transmission in vertebrates. Octopamine has been shown to inhibit ACh release from guinea-pig ileal synaptosomes but the results indicate that octopamine mediates its effects via  $\alpha_2$ -adrenoreceptors rather than octopamine receptors (Chang and Cheng, 1993). Adrenoreceptors are activated by the catecholamine, noradrenaline (norepinephrine) which is structurally related to octopamine and thought to be the vertebrate equivalent of octopamine. It is not

surprising, therefore, that there is evidence that noradrenaline modulates cholinergic neurons in vertebrates (Fort et al., 1995; Zarborsky and Cullinan, 1996; Smiley et al., 1999; Tellez et al., 1999). A number of pharmacological and anatomical studies provide evidence that noradrenaline modulates cholinergic neurons in the basal forebrain. EM studies have revealed cholinergic neurons in the rat and primate basal forebrain receive synaptic inputs from noradrenergic axons (Zarborsky and Cullinan, 1996; Smiley et al., 1999). Complementary to this are the results from an electrophysiological study (Fort et al., 1995), which revealed that cholinergic neurons from the guinea-pig basal forebrain respond to noradrenaline, indicating noradrenaline can potentially modulate ACh release. Indeed, a microdialysis study on the rat cerebral cortex revealed noradrenergic agonists and antagonists modulate ACh release (Tellez et al., 1999).  $\alpha_2$ -Adrenoreceptor antagonists increased the release of ACh and  $\alpha_2$ -adrenoreceptor agonists reduced the release of ACh, indicating that  $\alpha_2$ -adrenoreceptors located on the noradrenergic neurons and/or the cholinergic neurons themselves were involved in the down-regulation of ACh release from the cholinergic neurons.

An additional consequence of octopamine acting on the postsynaptic neuron, BA1, may be the modulation of synaptic transmission between the motoneuron BA1 and the first basalar depressor muscle. Transmission at this neuromuscular synapse is likely to be glutamatergic as all excitatory motoneurons in locust thoracic ganglia are thought to be glutamate-immunoreactive (Bicker et al., 1988; Watson and Seymour-Laurent, 1993) and glutamate has been shown to be released from the nerve terminals of locust motoneurons (Usherwood et al., 1968). There is also recent evidence that octopamine acts presynaptically to inhibit glutamatergic synaptic transmission at a neuromuscular junction in larval *Drosophila* (Nishikawa and Kidokoro, 1999). The octopaminergic

modulation of BA1 which has been shown in this study could therefore potentially result in glutamatergic transmission between BA1 and the first basalar depressor muscle being modulated.

#### **5.4.2. How does octopamine mediate its modulatory effect?**

The results presented in this chapter indicate that octopamine modulates synaptic transmission between the fSR and BA1 by suppressing the response of the postsynaptic neuron, BA1 to ACh. Octopamine may mediate its modulatory effects on BA1 via a number of different mechanisms. Possibilities include octopamine altering the membrane conductance of BA1, interfering with the second messenger systems in BA1, altering the ionic gradients across the membrane of BA1 or affecting the number of nicotinic cholinergic receptors available to bind ACh.

To determine if octopamine altered the membrane conductance of BA1, the input resistance and membrane potential of BA1 were monitored throughout the experiments. Following the bath application of octopamine ( $10^{-4}$  M) the input resistance of BA1 did not change significantly. However, the membrane potential of BA1 did depolarise initially following the application of octopamine ( $10^{-4}$  M). Octopamine-induced depolarisations of the membrane potential have also been recorded from locust flexor motoneurons (Sombati and Hoyle, 1984b; Parker, 1996) and from a cockroach inhibitory motoneuron (Pitman and Davis, 1988). Injection of octopamine into specific neuropil regions in the locust thoracic ganglia evoked 3-4 mV depolarisations in the membrane potential of the locust flexor motoneurons which returned to the resting potential after 0.3-2 minutes (Sombati and Hoyle, 1984b). Similarly, the membrane

potential of BA1 generally repolarised to resting potential levels within 10 minutes of application of octopamine. The amplitude of the cholinergic response of BA1 was still decreased when the membrane potential of BA1, returned to resting potential levels. This observation indicates that the effects on the cholinergic response of BA1 following octopamine application are not due to changes in the membrane conductance of BA1. Studying the effects of octopamine under voltage-clamp conditions may provide more conclusive evidence that octopamine does not cause a change in the membrane conductance of BA1. It is still possible, however, that octopamine may cause a conductance change in BA1 beyond the site of the voltage clamp.

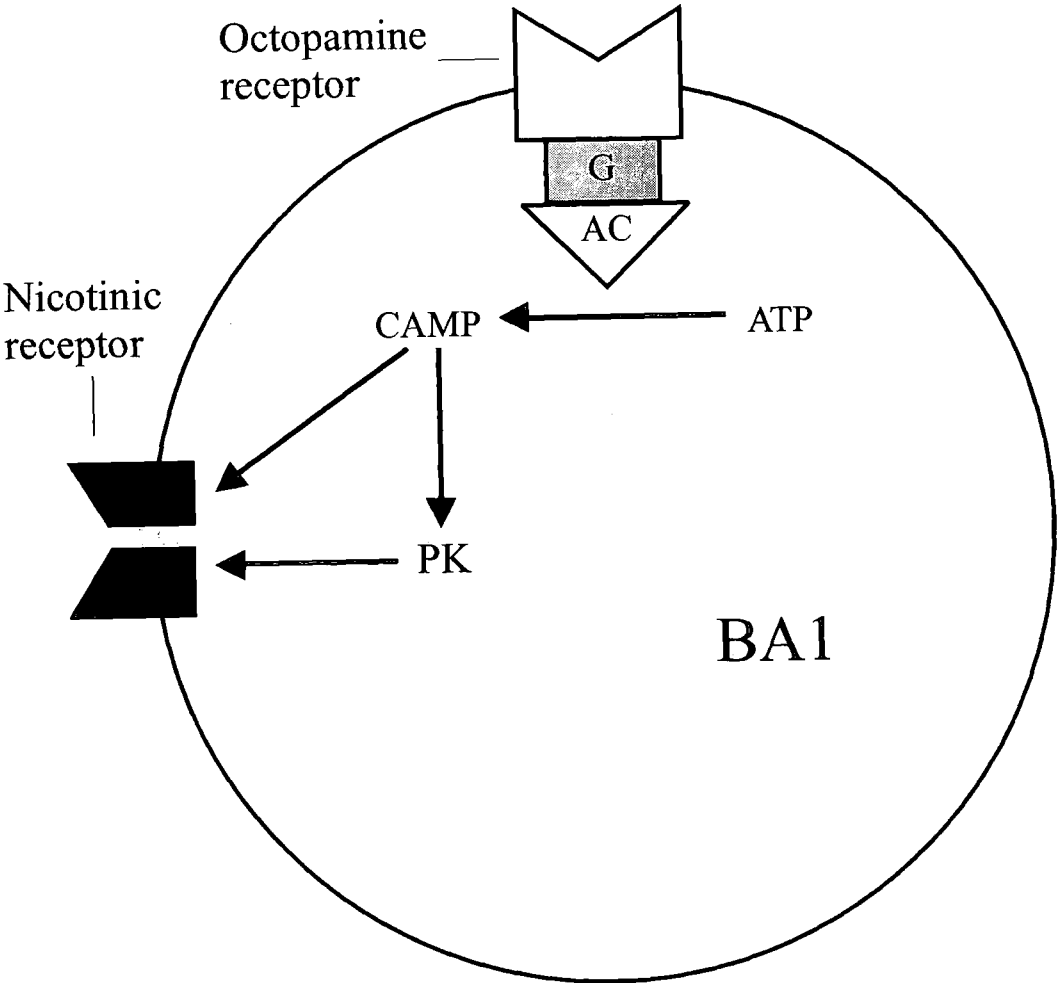
Another possibility is that octopamine modulates the cholinergic response of BA1 via octopamine receptors. Octopamine receptors are G-protein-coupled so therefore mediate their effects via second messenger systems. These second messenger systems may modulate the nicotinic cholinergic receptors responsible for the cholinergic response of BA1 (Figure 5.16). The majority of octopamine receptors identified are positively coupled to adenylate cyclase (Nathanson and Greengard, 1973; Nathanson, 1979; Uzzan and Dudai, 1982; Orchard et al., 1983; Evans 1984 a,b ; Orr and Hollingworth, 1990; Orr et al., 1991; Kaupp et al., 1992; Lange and Tsang, 1993; Roeder and Nathanson, 1993; Baines and Downer, 1994; Li et al., 1994; Han et al., 1998). In the adenylate cyclase pathway, adenylate cyclase converts ATP to cAMP which in turn activates protein kinases which phosphorylate certain proteins to alter their shape and functions. Octopaminergic stimulation of protein kinases and the phosphorylation of proteins by cAMP-dependent protein kinases (Edwards and Battelle, 1987; Hildebrandt and Müller, 1995; Battelle et al., 1998) demonstrates that octopamine is capable of modulating proteins via the adenylate cyclase pathway. There

**Figure 5.16. Model for the modulation of nicotinic cholinergic receptors by octopamine receptors via second messenger systems.**

Activation of G-protein-coupled octopamine receptors on BA1 may stimulate the enzyme adenylate cyclase (AC). The adenylate cyclase converts ATP to cAMP leading to an increase in intracellular cAMP levels. The elevated cAMP may modulate the nicotinic receptors, either directly or indirectly via protein kinases (PK), which phosphorylate the receptors.



**Figure 5.16. Model for the modulation of nicotinic cholinergic receptors by octopamine receptors via second messenger systems.**



is evidence in vertebrate preparations that neuronal nicotinic receptors can be phosphorylated and therefore modulated by protein kinases (Hunigar and Greengard, 1990; Swope et al., 1995; Eilers et al., 1997; Fenster, 1999). It is therefore possible that the activation of octopamine receptors stimulates protein kinases to phosphorylate and modulate nicotinic receptors in invertebrates. A recent study in an invertebrate preparation (Butt and Pitman, 1999) reported that protein phosphorylation was required for the amine 5HT to modulate nicotinic cholinergic responses recorded from the D<sub>f</sub> motoneuron in the cockroach. Therefore, octopamine may also modulate nicotinic cholinergic responses recorded from BA1 via protein phosphorylation.

A number of other octopamine receptors including some of those positively coupled to adenylate cyclase appear to be coupled to other second messenger systems. Activation of octopamine receptors on cultured cricket mushroom body neurons caused a decrease in intracellular calcium  $[Ca^{2+}]_i$  levels when the resting level of  $[Ca^{2+}]_i$  was approximately 126 nM (Cayre et al., 1999). If the resting level of  $[Ca^{2+}]_i$  was lowered to approximately 60 nM, however, octopamine receptor activation caused an increase in  $[Ca^{2+}]_i$  levels. An elevation in  $[Ca^{2+}]_i$  was also induced when cloned *Drosophila* octopamine/tyramine receptors and *Drosophila* octopamine receptors were stimulated (Robb et al., 1994; Reale et al., 1997; Han et al., 1998). Additionally, octopamine receptors on cockroach haemocytes and cloned *Lymnaea* octopamine receptors expressed in mammalian cells were reported to be coupled to an inositol triphosphate which usually causes an increase in  $[Ca^{2+}]_i$  (Baines and Downer, 1994; Gerhardt et al., 1997a). Another octopamine receptor, also cloned recently from *Lymnaea*, was reported to be coupled to a different second messenger system (Gerhardt et al., 1997b). Stimulation of this octopamine receptor did not induce changes in cAMP or inositol

phosphates but it did activate a chloride current, indicating it is coupled to chloride channels. It is not clear which if any of the mentioned second messenger systems are coupled to the octopamine receptors that may be present on the postsynaptic neuron BA1. However, this could be possibly determined with further experiments investigating the effects of calcium chelators and adenylate cyclase inhibitors on the octopaminergic modulation of the nicotinic cholinergic response of BA1.

#### **5.4.3. Characterisation of insect octopamine receptor classes**

One of the aims of this study was to investigate the characteristics of the receptors on BA1 which may mediate the octopaminergic modulation of the cholinergic response. The results from the experiments which examined the effects of insect octopamine receptor agonists on ACh responses suggest receptors on BA1 may be octopamine<sub>2A</sub> receptors. This could not be verified, however, by the results from the experiments which examined the effects of insect octopamine receptor antagonists on ACh responses. This was due to a number of reasons.

Unfortunately, the compounds available to characterise insect octopamine receptors are not specific for octopamine receptors. The antagonists which have been used to characterise octopamine receptors in invertebrate preparations (Evans, 1981; Nathanson, 1985; Saudou et al., 1990; Orr et al., 1991; Roeder, 1992, 1995) also act at a number of other amine receptors in both vertebrates and invertebrates. The majority of antagonists used act at vertebrate  $\alpha$ -adrenoreceptors. Others, for example, metoclopramide act at vertebrate dopamine receptors. Even epinastine, which has been reported recently to be a highly specific antagonist of insect octopamine receptors, acts

at the vertebrate histamine receptor (Roeder et al., 1998). Some of these compounds have also been reported to act at other amine receptors in insect preparations (Pitman and Davis, 1988). Therefore, the antagonists used to characterise the octopamine receptors which may be present on BA1 may be acting at other amine receptors in the locust.

The antagonists used to characterise potential octopamine receptors on BA1 also had complex actions on the cholinergic response of BA1. The antagonists had a purely agonistic rather than antagonistic action on ACh responses recorded from BA1. They mimicked the effects of octopamine and caused a decrease in the ACh response amplitude. This has been reported previously in the study on the locust extensor-tibiae preparation (Evans, 1981). The insect octopamine receptor antagonist metoclopramide acted as a partial agonist at the octopamine receptors on the extensor-tibiae muscle. The agonistic action on the cholinergic responses of BA1 made it difficult to examine any antagonistic effect the insect octopamine receptor antagonists may have had. When the antagonists phentolamine and metoclopramide were applied at concentrations low enough not to have an agonistic effect, they did not appear to antagonise the inhibitory action of octopamine on the ACh responses. Similar findings have been reported in a study on the effects of octopamine on crayfish promotor motoneuron activity (Gill and Skorupski, 1996). The inhibitory action of octopamine on the motoneuron activity was only weakly antagonised by phentolamine. As the insect octopamine receptor antagonists at the concentrations tested do not appear to antagonise the inhibitory effects of octopamine on ACh responses recorded from BA1, the receptors that may be involved in the octopaminergic modulation of ACh responses cannot be characterised.

#### 5.4.4. Presynaptic receptors

The results presented in this chapter show that octopamine causes a decrease in the electrically-evoked EPSP amplitude recorded from BA1, whereas the  $\alpha$ -adrenergic antagonists phentolamine and mianserin cause an increase in the electrically-evoked EPSP amplitude. Octopamine and phentolamine have also been reported to have opposite effects in a recent study on crayfish rhythmic motor output (Gill and Skorupski, 1999). It was suggested by Gill and Skorupski (1999) that the excitatory effect of phentolamine on the motor activity was due to the antagonist blocking the inhibitory effects of endogenous octopamine on the motor output. It is possible, therefore, that the increase in synaptic transmission at the fSR/BA1 synapse following  $\alpha$ -adrenergic antagonist application is due to the inhibitory effects of endogenous octopamine on the fSR/BA1 synapse being blocked. The increase in the response of BA1 to the electrical stimulation of the fSR, is not due to the  $\alpha$ -adrenergic antagonists phentolamine and mianserin acting on the postsynaptic neuron BA1 however, as previous experiments demonstrated that phentolamine and mianserin cause a decrease in the cholinergic response of BA1. This indicates the increase in the EPSP amplitude is due to phentolamine and mianserin acting presynaptically.

The presynaptic mechanisms that mediate the effects of phentolamine and mianserin are unclear from the experiments presented in this chapter. The  $\alpha$ -adrenergic antagonists may block presynaptically located octopamine receptors involved in the regulation of ACh release from the fSR terminals or there may be another explanation. There is evidence that at high concentrations the  $\alpha$ -adrenergic antagonist, phentolamine has a local anaesthetic action on excitable tissues by blocking sodium channels

(Ramirez and French, 1990). A study on the anaesthetic effect of phentolamine on the fSR (Ramirez and Pearson, 1990) revealed that phentolamine ( $3 \times 10^{-4}$  M) abolished the response of the fSR to mechanical stimulation after approximately 10 minutes. Before this inactivation, however, the fSR repetitively discharged. This could potentially cause more ACh to be released from the fSR terminals which would cause an increase in the amplitude of the electrically-evoked EPSPs recorded from BA1. Phentolamine only caused the fSR to repetitively discharge, however, in experiments in which the fSR was mechanically stimulated. Phentolamine did not cause the nerve, containing the fSR axon (nerve 1D) to repetitively discharge when it was isolated. This indicates that phentolamine mediates this effect peripherally, at the site where the fSR initiates spikes in response to the wing being raised. Therefore, phentolamine should not cause the fSR to repetitively discharge, in the preparation used in this study.

The increase in the electrically-evoked EPSP amplitude may also be due to the  $\alpha$ -adrenergic antagonists blocking presynaptically located octopamine receptors involved in the down-regulation of ACh release from the fSR terminals. There is evidence that octopamine acts at presynaptic terminals to modulate neurotransmitter release in other invertebrate preparations (Evans, 1981; Breen and Atwood, 1983; Howells and Evans, 1998; Nishikawa and Kidokoro, 1999). Octopamine was reported to increase neurotransmitter release at a neuromuscular junction in crayfish (Breen and Atwood, 1983), whereas octopamine was reported to presynaptically inhibit neuromuscular transmission in *Drosophila* (Nishikawa and Kidokoro, 1999). At the locust extensor-tibiae neuromuscular junction, two types of presynaptic octopamine receptors have been identified (Evans, 1981; Howells and Evans, 1998). Octopamine heteroreceptors, located on the presynaptic terminals of the slow motoneuron to the tibiae muscle in the

locust hind leg are reported to up-regulate transmitter release from the presynaptic terminals, which increases the amplitude of twitch tension in the extensor-tibiae muscle (Evans, 1981). These octopamine receptors were characterised as octopamine<sub>2A</sub> receptors. Octopamine autoreceptors, located on the presynaptic terminals of the DUM neuron DUMETi, which innervates the extensor-tibiae neuromuscular junction, were reported to down-regulate the release of octopamine from the presynaptic terminals (Howells and Evans, 1998). The octopamine autoreceptors on DUMETi were closer pharmacologically to octopamine<sub>2</sub> receptors than octopamine<sub>3</sub> receptors.

If the increase in the EPSP amplitude recorded from BA1 is due to  $\alpha$ -adrenergic antagonists blocking presynaptically located octopamine receptors, then the results indicate that the octopamine receptors located presynaptically are of a different class to the octopamine receptors located on BA1. None of the insect octopamine receptor antagonists tested appeared to antagonise potential octopamine receptors on BA1. The results from experiments examining the effects of the antagonists on EPSPs, however, indicate that potential octopamine receptors located presynaptically appear to be sensitive to the  $\alpha$ -adrenergic antagonists, mianserin and phentolamine and insensitive to the dopamine antagonist, metoclopramide. The pharmacological profile of potential octopamine receptors located presynaptically at the fSR/BA1 synapse is most closely related to the neuronal octopamine<sub>3</sub> receptor profile (see Table 5.1).

Different classes of pre- and postsynaptic octopamine receptors have been reported at the locust extensor-tibiae neuromuscular junction (Evans, 1981; Howells and Evans, 1998). Similar findings have also been reported in vertebrate preparations. The results from a recent microdialysis study (Tellez et al., 1999), examining the effects  $\alpha_2$ -

adrenoreceptor agonists and antagonists on ACh release from the rat cortex, indicate that different types of  $\alpha_2$ -adrenoreceptors are present at synapses between noradrenergic afferents and cholinergic cortical neurons. The results suggest that  $\alpha_2$ -adrenergic autoreceptors are located presynaptically on the noradrenergic afferents and down-regulate noradrenaline release, whereas both inhibitory  $\alpha_2$ -adrenergic heteroreceptors and excitatory non- $\alpha_2$ -adrenoreceptors are located on the cholinergic neurons.

If octopamine receptors are located presynaptically at the fSR/BA1 synapse, there are a number of different neurons that the receptors may be located on. The octopamine receptors may be located on the fSR itself and directly modulate ACh release. A previous study (Ramirez and Orchard, 1990), has reported that the fSR is modulated by octopamine, indicating that the fSR does possess octopamine receptors. Octopamine caused an increase in the response of the fSR to wing movements. Both the number and frequency of spikes recorded from the fSR increased following octopamine application and this was antagonised by phentolamine. The recordings were made from the peripheral nerve, however, so the results do not provide conclusive evidence that octopamine receptors are located on the presynaptic terminals of the fSR within the CNS. Recordings would have to be made from the presynaptic terminals of the fSR itself as well as any other neurons that may possess the receptors to establish the presence and characteristics of the presynaptic octopamine receptors, although this would be very technically difficult.

Presynaptic octopamine receptors could also be located on the octopaminergic neuron where they could act as autoreceptors. Some of the possible locations of octopamine



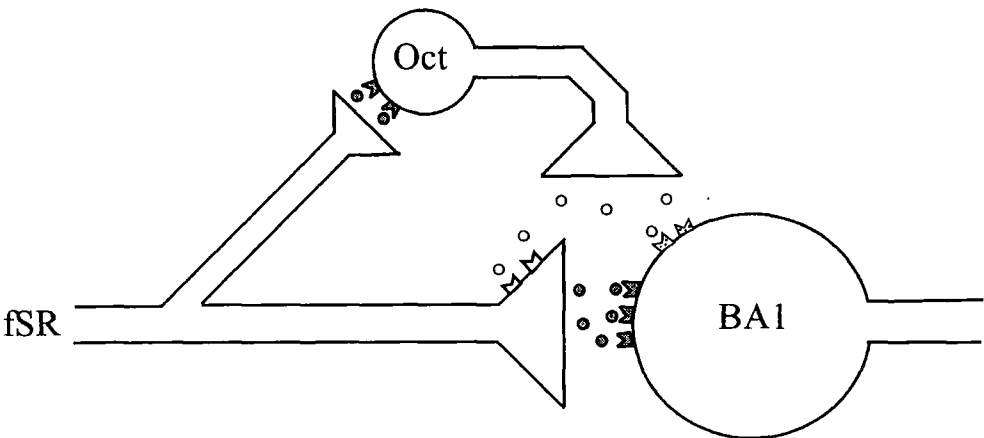
receptors at the fSR/BA1 synapse are shown on models in Figure 5.17. The diagrams, which show possible pathways mediating the octopaminergic modulation of the fSR/BA1 synapse, are based on the results reported in this chapter. It is suggested that when the fSR is stimulated either electrically or by the wing being raised and ACh is released from its terminals, some may bind to receptors on an octopaminergic neuron (possibly DUM) which receives a collateral input from the fSR, as well as to cholinergic receptors on BA1, evoking a cholinergic response. Activation of the octopaminergic neuron would cause the release of octopamine which could potentially bind to octopamine receptors on the postsynaptic neuron, BA1 and reduce the cholinergic response of BA1. Octopamine may reduce the cholinergic response of BA1 by altering the membrane conductance or ionic gradients of BA1, by interfering with the second messenger systems, by affecting the number of nicotinic receptors available to bind ACh or by acting at octopamine receptors on BA1. Inhibition of the cholinergic response of BA1 by octopamine has been demonstrated in the experiments in this chapter. The octopamine may also bind to receptors located presynaptically.

In model 1, inhibitory octopamine heteroreceptors located on the fSR terminals inhibit the release of ACh. (Figure 5.17Ai). Application of  $\alpha$ -adrenergic antagonists have little or no effect on the postsynaptic octopamine receptors but block the octopamine heteroreceptors on the fSR terminals, causing more ACh to be released (Figure 5.17Aii). This is consistent with the results in this chapter which show that application of  $\alpha$ -adrenergic antagonists causes an increase in the amplitude of the electrically-evoked EPSPs.

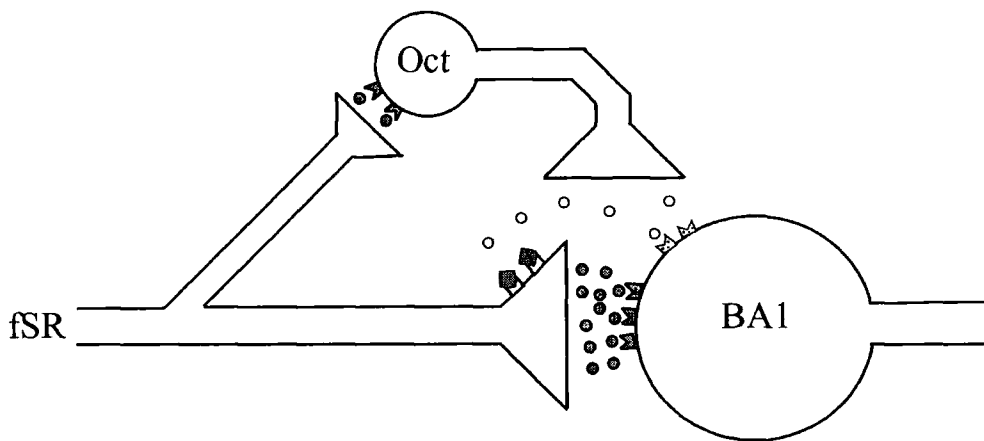
**Figure 5.17A. Modulation of the fSR/BA1 synapse by octopamine receptors. Model 1.** (i) In the absence of antagonists. Stimulation of the fSR axon, results in the release of acetylcholine (ACh) from the fSR terminals. The ACh binds to cholinergic receptors on BA1, evoking EPSPs and to cholinergic receptors on an octopaminergic neuron. Stimulation of the octopaminergic neuron results in the simultaneous release of octopamine in the vicinity of the fSR/BA1 synapse. The octopamine binds to inhibitory octopamine receptors on BA1, causing a decrease in the cholinergic response of BA1 and to inhibitory octopamine heteroreceptors on the presynaptic terminals of the fSR, causing the release of ACh to be down-regulated. (ii) In the presence of  $\alpha$ -adrenergic antagonists. The blockade of the inhibitory octopamine receptors on the presynaptic terminals of the fSR by  $\alpha$ -adrenergic antagonists, prevents the normal down-regulation of ACh release. Therefore, more ACh is released resulting in an increase in the amplitude of the EPSPs recorded from BA1.

**Figure 5.17A. Modulation of the fSR/BA1 synapse by octopamine receptors. Model 1.**

(i) In the absence of antagonists.



(ii) In the presence of  $\alpha$ -adrenergic antagonists.



- Acetylcholine
- Octopamine
- Nicotinic receptor
- ▣ Inhibitory Octopamine heteroreceptor sensitive to  $\alpha$ -adrenergic antagonists
- ▣ Inhibitory Octopamine receptor insensitive to  $\alpha$ -adrenergic antagonists
- ◆  $\alpha$ -adrenergic antagonist

In model 2, inhibitory octopamine autoreceptors sensitive to  $\alpha$ -adrenergic antagonists are located on the octopaminergic neuron and excitatory octopamine heteroreceptors insensitive to  $\alpha$ -adrenergic antagonists are located on the fSR terminals. The inhibitory octopamine autoreceptors on the octopaminergic neurons presynaptic terminals down-regulate the release of octopamine whereas the excitatory octopamine heteroreceptors on the fSR presynaptic terminals increase the release of ACh (Figure 5.17Bi).

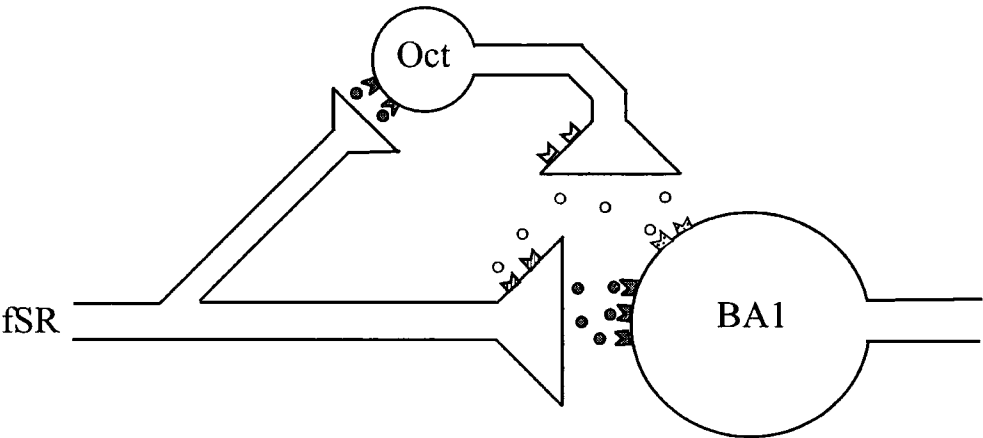
Application of  $\alpha$ -adrenergic antagonists has little or no effect on the octopamine receptors on the postsynaptic neuron or fSR terminals but blocks the inhibitory octopamine autoreceptors. This causes more octopamine to be released which activates the excitatory octopamine heteroreceptors on the fSR terminals causing more ACh to be released (Figure 5.17Bii). Again, this is consistent with the results in this chapter which show that application of  $\alpha$ -adrenergic antagonists causes an increase in the amplitude of the electrically-evoked EPSPs. These models propose, therefore, that different classes of octopamine receptors ( $\alpha$ -adrenergic sensitive and insensitive) that have either excitatory or inhibitory effects are present at the synapse between the fSR and BA1. Different octopamine receptor classes which mediate different effects at the same synapse have been reported at the locust extensor-tibiae neuromuscular junction (Evans, 1981; Howells and Evans, 1998) and evidence of both excitatory non- $\alpha_2$ -adrenoreceptors and inhibitory  $\alpha_2$ -adrenoreceptors at the synapse between noradrenergic afferents and cholinergic cortical neurons in the rat forebrain has been reported (Tellez et al., 1999).

An additional possibility is that the octopamine receptors are located on another neuron e.g. a GABAergic interneuron, which when activated by octopamine, releases GABA onto the fSR terminals to inhibit the release of ACh. In the presence of  $\alpha$ -adrenergic

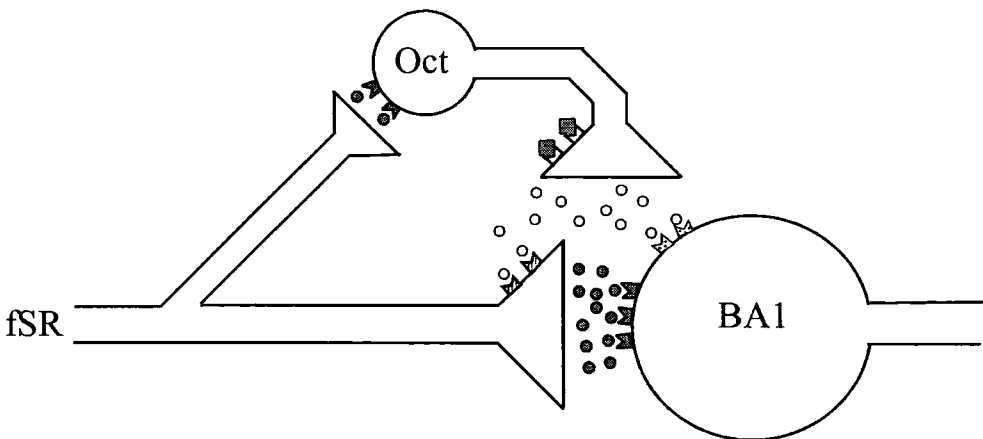
**Figure 5.17B. Modulation of the fSR/BA1 synapse by octopamine receptors. Model 2.** (i) In the absence of antagonists. Stimulation of the fSR axon, results in the release of acetylcholine (ACh) from the fSR terminals, which binds to cholinergic receptors on BA1 and on an octopaminergic neuron. Stimulation of the octopaminergic neuron results in the simultaneous release of octopamine in the vicinity of the fSR/BA1 synapse. The octopamine binds to inhibitory octopamine receptors on BA1, causing a decrease in the cholinergic response of BA1 and to octopamine receptors located presynaptically. These include inhibitory octopamine autoreceptors on the presynaptic terminals of the octopaminergic neuron, which down-regulate the release of octopamine and excitatory octopamine heteroreceptors on the presynaptic terminals of the fSR, which up-regulate the release of ACh. (ii) In the presence of  $\alpha$ -adrenergic antagonists. The blockade of the inhibitory octopamine autoreceptors on the presynaptic terminals of the octopaminergic neuron by  $\alpha$ -adrenergic antagonists, prevents the normal down-regulation of octopamine release. Therefore, more octopamine is released, which binds to excitatory octopamine heteroreceptors on the presynaptic terminals of the fSR, causing more ACh to be released. This in turn leads to an increase in the amplitude of the EPSPs recorded from BA1.

**Figure 5.17B. Modulation of the fSR/BA1 synapse by octopamine receptors. Model 2.**

(i) In the absence of antagonists.



(ii) In the presence of  $\alpha$ -adrenergic antagonists.



- |                      |  |
|----------------------|--|
| • Acetylcholine      | ○ Octopamine   |
| ■ Nicotinic receptor | ■ Inhibitory Octopamine receptor insensitive to $\alpha$ -adrenergic antagonists       |
|                      | ■ Inhibitory Octopamine autoreceptor sensitive to $\alpha$ -adrenergic antagonists     |
|                      | ▨ Excitatory Octopamine heteroreceptor insensitive to $\alpha$ -adrenergic antagonists |
|                      | ◆ $\alpha$ -adrenergic antagonist  |

antagonists, the GABAergic interneuron would not be activated so the inhibition on the fSR terminals would be lifted, causing more ACh to be released. The results presented in Chapters 3 and 4 indicate that the fSR terminals do receive synaptic inputs from GABA-immunoreactive neurons which appear to be involved in the modulation of ACh release from the fSR terminals. Interestingly, there is evidence that indicates that the GABA-mediated presynaptic inhibition of other sensory afferents increases in the presence of octopamine (Matheson, 1997). FeCO sensory neurons in the locust receive presynaptic inhibitory inputs onto their central terminals (Burrows and Laurent, 1993; Burrows and Matheson, 1994). One of the explanations for this presynaptic inhibition is that the sensory neurons from the FeCO activate GABAergic interneurons which in turn inhibit the central terminals of the sensory neurons from the same organ, and so regulate the sensory information to postsynaptic neurons (Burrows and Laurent, 1993; Burrows and Matheson, 1994; Wolf and Burrows, 1995). Application of octopamine increased the presynaptic inhibition received by FeCO sensory neurons coding for the position of the tibiae but not by those that code for the movement of the tibiae (Matheson, 1997). It is also worth noting that there is evidence that indicates an inhibitory GABAergic interneuron is present in the pathway between the tegula afferents and the octopaminergic DUM neurons which innervate flight muscles in the locust (Duch and Pflüger, 1999).

#### **5.4.5. Source of octopamine**

One possible source of octopamine which could potentially modulate the fSR/BA1 synapse is the octopaminergic DUM neurons which have been shown to be involved in locust flight (Whim and Evans, 1988; Ramirez and Orchard, 1990; Stevenson and

Meuser, 1997; Duch and Pflüger, 1999). The results from the confocal microscopical immunocytochemical study indicate the octopamine-immunoreactivity present in the dorso-lateral neuropil region where it could modulate the fSR/BA1 synapse corresponds with the known projections of the DUM neuron processes within the locust thoracic ganglia (Watson, 1984).

The distribution of octopamine-immunoreactivity in the mesothoracic ganglion revealed in the confocal microscopical study in this chapter was consistent with the location of octopaminergic neurons revealed in other studies, confirming that the anti-octopamine antibody used is specific for octopamine (Stevenson et al., 1992; Stevenson and Spörhase-Eichmann, 1995). The mesothoracic ganglion possesses one pair of ventral lateral octopaminergic neurons and twenty one DUM neurons (Stevenson et al., 1992; Duch et al., 1999). The somata of both the ventral lateral neurons and the DUM neurons are located posterior to the emergence of nerve 5, explaining why no octopamine-immunoreactive cell bodies were evident on the transverse slices taken at the levels of nerve 3 and nerve 5. The cell bodies of the ventral neurons each give rise to a primary neurite which extends anteriorly (Stevenson et al., 1992). The small region of dense octopamine-immunoreactivity close to the ventral lateral edge of the slices taken at the level of nerve 5 (Figure 5.15B) may represent a number of fine processes projecting from one of these ventral lateral cells. The dense octopamine-immunoreactivity along the dorsal edge of transverse slices taken at the levels of nerve 3 and 5 is likely to be the lateral neurites of octopaminergic DUM neurons. The majority of octopaminergic DUM neurons have a similar gross morphology. The soma of each DUM neuron positioned in the dorsal midline gives rise to a primary neurite which extends anteriorly before bifurcating into two bilateral processes, forming a



characteristic T-junction (Watson, 1984). The bilateral processes give rise to numerous neurites which extend into the neuropil regions of the thoracic ganglia and one or more axons which extend along the dorsal edge of the ganglion into the lateral nerves.

Therefore, the distribution of octopamine-immunoreactivity along the dorsal edge of the mesothoracic ganglion and within the dorso-lateral neuropil regions revealed in this and previous studies corresponds with the processes of the DUM neurons (Watson, 1984). As the processes of the DUM neurons project into the same dorso-lateral neuropil region into which the fSR and BA1 project, they are strong candidates as the source of the octopamine which potentially modulates the fSR/BA1 synapse.

DUM neurons have been implicated as the source of octopamine in the modulation of numerous other preparations. Many studies including this one implicate the DUM neurons as the source of octopamine on the basis of their proximity to the neurons or muscles which are modulated (Sombati and Hoyle 1984 a, b; Bräunig, 1997; Stevenson and Meuser, 1997; Bräunig and Eder, 1998). Others have revealed that stimulation of the DUM neurons mimics the modulatory action of octopamine and this is blocked by octopamine receptor antagonists (Evans, 1981; Sombati and Hoyle 1984a, b; Whim and Evans, 1988; Ramirez and Orchard, 1990; Stevenson and Meuser, 1997; Bräunig and Eder, 1998). The DUM neurons may mediate their effects either directly by innervating their targets (Bräunig and Eder, 1998) or indirectly by releasing octopamine into the haemolymph (Ramirez and Orchard, 1990). Octopamine may be released into the haemolymph by being released onto skeletal muscles initially (Evans, 1981, Bräunig et al., 1994; Bräunig 1997; Stevenson and Meuser, 1997) and then diffusing into the haemolymph or being released from neurohaemal release sites. Evidence of these sites has been shown recently in studies on octopaminergic neurons in both the locust and

*Manduca sexta* (Bräunig et al., 1994; Bräunig, 1997; Consoulas et al., 1999). DUM1b neurons form a meshwork of varicose endings on the surfaces of sympathetic nerves and DUM 3,4,5 neurons form neurohaemal release sites on the surfaces of peripheral nerves (Bräunig et al., 1994; Bräunig, 1997). Although, the direct peripheral innervation of sensory neurons by DUM neurons has been reported recently (Bräunig and Eder, 1998) there are no reports of the direct innervation of central neurons by DUM neurons. DUM neurons have been shown to be involved in the modulation of central neurons (Sombati and Hoyle, 1984 a,b) but there is a lack of evidence showing the direct innervation of central neurons by DUM neurons. An electron-microscopical study by Watson (1984) revealed that locust DUM neurons possessed very few conventional synaptic outputs. A number of unusual presynaptic densities were observed, however, that were not associated with synaptic vesicles. Interestingly, in vertebrates, neurons immunoreactive for noradrenaline, the monamine structurally related to octopamine, also possess membrane specialisations that can not be confidently identified as synapses (Umbriaco et al., 1995; Smiley et al., 1999). The synaptic outputs that were positively identified in the spines of some locust DUM neurons were associated with clear vesicles, 40-60 nm in diameter (Watson, 1984). The description of the vesicles is very similar to those present in some of the non GABA-immunoreactive neurons that are presynaptic to the fSR, revealed in Chapter 3 although the very presence of clear synaptic contacts in these non GABA-immunoreactive neurons suggests that they are not DUM neurons. It is still possible that these and other non GABA-immunoreactive neurons in close contact with the fSR/BA1 synapse are octopaminergic DUM neurons.

The number of octopaminergic DUM neurons which could potentially be involved in the modulation of the fSR/BA1 synapse modulation of the fSR/BA1 synapse is unclear. However, recent work has shown that only subpopulations of DUM neurons rather than a whole population are involved in specific types of behaviour (Burrows and Baudoux, 1998; Duch and Pflüger, 1999; Duch et al., 1999). It was previously thought that whole populations of DUM neurons were recruited during certain types of behaviour to mediate general arousal (Ramirez and Orchard, 1990; Orchard et al., 1993; Parker, 1996). However, recent studies have revealed only specific subsets of DUM neurons are activated rather than whole populations during certain behaviours, which is consistent with the 'Orchestration hypothesis' proposed by Sombati and Hoyle (1984 b). According to this hypothesis, different subclusters of modulatory neurons are activated to initiate different specific behaviours.

The efferent DUM neurons in the locust thoracic ganglia can be divided into different subsets according to: the nerves they project into; and their peripheral targets; their presynaptic inputs (Baudoux and Burrows, 1998; Duch and Pflüger, 1999; Duch et al., 1999). Efferent DUM neurons are identified on the basis of the nerves they project into (e.g. DUM 3, 4 neuron projects into nerve 3 and nerve 4). The peripheral targets of DUM 3 and DUM 3,4 neurons are flight power muscles (Kutsch and Schneider, 1987; Duch et al., 1999) and the peripheral targets of DUM 3,4,5 and DUM 5 neurons are leg muscles (Braünig et al., 1997; Duch and Pflüger, 1999). Interestingly, these two subsets of DUM neurons are differentially activated (Baudoux and Burrows, 1998; Duch and Pflüger, 1999; Duch et al., 1999). The DUM neurons which innervate flight muscles are not affected by wind stimuli but those that innervate leg muscles are (Duch and Pflüger, 1999). Also, electrical stimulation of tegula afferents, which monitor wing

position, results in IPSPs in the DUM neurons which innervate flight muscles, but no response in the DUM neurons that innervate leg muscles. This inhibition of DUM neurons by the tegula afferents may contribute to this subset of DUM neurons being inhibited during flight. The DUM neurons which innervate flight muscles (DUM 3A and DUM 3,4), were inhibited most strongly during each wing depressor phase of the flight sequence and they also received inhibitory inputs during octopamine-induced fictive flight patterns. On the other hand, DUM neurons which innervated leg muscles (DUM 3,4,5 and DUM 5) were strongly excited, and produced spikes during a flight sequence and during octopamine-induced fictive flight patterns. These results clearly demonstrate that DUM neurons innervating leg muscles (DUM 5 and DUM 3,4,5) are differentially activated to DUM neurons innervating flight muscles (DUM 3 and DUM3,4) during flight.

The inhibition of the DUM 3 and DUM 3,4 neurons during flight revealed in the study by Duch and Pflüger (1999) is in contrast to the findings reported by Ramirez and Orchard (1990), who reported that all types of DUM neurons were activated during flight. Duch and Pflüger (1999) suggested that the inhibition of one subset of DUM neurons and activation of another was to control the octopamine levels in the haemolymph. The raised octopamine levels in the haemolymph during the first 10 minutes of flight, which may be due to the excitation of the DUM neurons with neurohaemal release sites, prevents muscle metabolism in the muscle switching from trehalose to lipid. They suggested that the decrease in octopamine concentration in the haemolymph after 10 minutes of flight was then due to the DUM neurons being inhibited. The decrease in octopamine would switch the metabolism in the muscle from trehalose to lipid which would be needed for prolonged flight. The reason, therefore, for

differentially activating DUM neurons in this behavioural context was to regulate octopamine levels in the haemolymph and control muscle metabolism for different periods of flight.

#### **5.4.6. Behavioural significance**

Before any hypotheses about the behavioural significance of the octopaminergic modulation of the fSR/BA1 can be made, the results from the experiments presented in this chapter have to be considered carefully. It is possible the experimental conditions may not represent the true physiological conditions, so although octopamine is capable of modulating the fSR/BA1 synapse under experimental conditions it may not *in vivo*. The experiments were performed on an isolated ganglia preparation rather than an intact animal, which could potentially affect the results. However, this may not be a problem as it was reported in another study (Ramirez and Orchard, 1990) that there was no difference in the effects of octopamine recorded from an isolated ganglia preparation or an intact animal. Another consideration is that the effects are mediated by drugs applied to, rather than by neurotransmitters released within, the preparation. It is possible that the concentration of octopamine shown to modulate the synapse is not physiologically relevant. The threshold concentration at which octopamine had an effect on the EPSPs and cholinergic responses recorded from BA1 was  $10^{-5}$  M. Other studies that have adopted protocols involving octopamine being applied to the surface of the thoracic ganglia used concentrations from  $10^{-5}$  M -  $5 \times 10^{-3}$  M (Mercer and Erber, 1983; Ramirez and Pearson, 1991; Parker, 1996; Weisel-Eichler and Libersat, 1996). It is unclear what the concentration of octopamine was that reached the fSR/BA1 synapse within the ganglia, although it has been reported that bath application of

octopamine ( $1.5 \times 10^{-3}$  M) results in central effects similar to those caused by injection of octopamine ( $10^{-6}$  -  $10^{-4}$  M) into the neuropil (Ramirez and Pearson, 1991).

Therefore, less than  $10^{-5}$  M octopamine can modulate the fSR/BA1 synapse. Whether or not this concentration is physiologically relevant is uncertain, although it is within the range of the concentrations used in other studies (Mercer and Erber, 1983; Sombati and Hoyle (1984 a,b); Ramirez and Pearson, 1991; Parker, 1996; Weisel-Eichler and Libersat, 1996).

In this study, the net effect of octopamine was to decrease cholinergic synaptic transmission between the sensory neuron, fSR and the motoneuron, BA1. A previous study (Ramirez and Orchard, 1990), reported that octopamine enhanced the response of the fSR to wing movements by increasing both the number and frequency of spikes, which could potentially increase cholinergic synaptic transmission between the fSR and BA1. The effects of octopamine at the fSR/BA1 synapse, therefore, appear to counteract the effects of octopamine on the fSR. Octopamine has been reported to have similar contradictory effects on another locust sensory neuron (Matheson, 1997).

Octopamine caused an increase in the spike frequency of the FeCO sensory neurons which code for the position of the locust tibia but also caused an increase in the amplitude of presynaptic inhibitory inputs to the central terminals of the same neurons. This indicates that the increase in sensory spiking is counteracted by the increase in presynaptic inhibition. It was suggested this could be a mechanism to balance sensory information before it is integrated in to the CNS. The sensory neurons of the FeCO are thought to be presynaptically inhibited by other sensory neurons from the same organ via interposing interneurons. It is possible that the increase in spiking caused by

octopamine may not be counteracted by an increase in presynaptic inhibition in all the sensory neurons resulting in a shift in the balance of information.

However, this explanation cannot account for the apparently counteractive effects of octopamine on the fSR/BA1 sensory pathway, as the fSR is a single sensory neuron. The counteractive effects of octopamine on the fSR/BA1 sensory pathway may be due to the octopamine which enhances the response of the fSR to wing movements and the octopamine which may inhibit transmission at the fSR/BA1 synapse, coming from alternate sources. The octopamine which increases the response of the fSR to wing movements appears to be from the haemolymph rather than directly from a DUM neuron (Ramirez and Orchard, 1990; Bräunig, 1997). Injection of octopamine into the haemolymph in the intact preparation caused an increase in the response of the fSR to wing movements (Ramirez and Orchard, 1990). Also, examination of the peripheral branching patterns of the DUM neurons, revealed that they do not project into the nerve where they could directly innervate the fSR peripherally (Bräunig, 1997). The octopamine which potentially inhibits synaptic transmission between the fSR and BA1, revealed in this study, is more likely to be directly from the neurites of DUM neurons within the CNS. The octopamine has to be present in the dorso-lateral neuropil region where it could potentially modulate the fSR/BA1 synapse and neurites of the DUM neurons have been reported to be present in this region (Watson, 1984). Different subsets of DUM neurons have been shown recently to be activated for specific behaviours (Baudoux and Burrows, 1998; Duch and Pflüger, 1999; Duch et al., 1999). It is possible therefore, that one set of DUM neurons may be involved in increasing by increasing the the response of the fSR and another set may be involved in suppressing synaptic transmission between the fSR and BA1.

DUM 3,4,5 neurons which possess neurohaemal release sites (Bräunig, 1997) are strongly excited and produce spikes during flight sequences and during octopamine-induced fictive flight patterns (Duch and Pflüger, 1999). Therefore, activation of these neurons during flight could potentially cause an increase in the octopamine levels in the haemolymph. An increase in the octopamine levels in the haemolymph could cause an increase in the response of the fSR to wing movement (Ramirez and Orchard, 1990), which would potentially increase transmission at the fSR/BA1 synapse. However, DUM 3 and DUM 3,4 neurons which innervate flight power muscles and which are inhibited during flight sequences, may be involved in decreasing transmission at the fSR/BA1 synapse. At rest all DUM neurons spontaneously spike, indicating they continuously release octopamine. If this subset of DUM neurons continuously release octopamine onto the fSR/BA1 synapse, cholinergic synaptic transmission will be continuously suppressed at rest. However, after the onset of flight when this subset of DUM neurons are inhibited, octopamine would no longer be released and cholinergic synaptic transmission would be enhanced. Therefore, if these suggestions are correct, the response of the fSR to wing movements and synaptic transmission between the fSR and BA1 will be enhanced, during flight.

Further work is required to provide conclusive evidence that a) pre- and postsynaptic octopamine receptors of different classes are involved in the modulation of the fSR/BA1 synapse during flight and that b) DUM neurons modulate the fSR/BA1 synapse in a behavioural context. However, the suggestions made in this discussion to explain the possible modulatory mechanisms and the behavioural significance of the octopaminergic modulation of fSR/BA1 synapse are based on the results reported in



this chapter and supported by information currently known on the roles and actions of octopamine.

## **Chapter 6. Modulation of the fSR/BA1 synapse by 5HT and dopamine.**

### **6.1. INTRODUCTION**

Biogenic amines, as mentioned in the previous chapter, are present in the CNS of both vertebrates and invertebrates and can act as neuromodulators to modify the neurons which control certain behaviours. 5-Hydroxytryptamine (5HT, also known as serotonin) and 3-hydroxytyramine (also known as dopamine) are two such biogenic amines.

5HT and dopamine have been shown to be widely distributed throughout invertebrate CNS (see Table 6.1), which suggests that these biogenic amines play an important role. This suggestion is supported by studies which have been shown that 5HT and dopamine modulate and generate numerous different behaviours in invertebrates including aggression (Livingstone et al., 1980), escape responses (Glanzman and Krasne, 1983; Casagrand and Ritzmann, 1992a) and learning (Brunelli et al., 1976; Mercer and Menzel, 1982; Byrne and Kandel, 1996). As the neurons which control certain behaviours in invertebrates have been identified, the neural circuitry underlying the amines affects on behaviour can also be understood by examining the effects of these amines on neuronal pathways (Bicker and Menzel, 1989).

For example, there appears to be a direct relationship between the actions of 5HT and dopamine on the neurons involved in the escape response in the cockroach and crayfish and the effects these neuromodulators have on the escape reflex. The lateral giant escape reaction in the crayfish is evoked when mechanosensory receptors on the tail fan

**Table 6.1. The distribution of 5HT and dopamine in the CNS of different invertebrate species.**

Species	5HT	Dopamine
Locust	Tyrer et al., 1984, Lutz and Tyrer, 1988	Vieillemaringe et al., 1984, Watson, 1992b, Wendt and Homberg, 1992
Cockroach	Bishop and O'Shea, 1983	Distler, 1990
Honey bee	Mercer et al., 1983, Rehder et al., 1987	Mercer et al., 1983, Schäfer and Rehder, 1989
Cricket	Elekes et al., 1987, Hörner et al., 1995	Elekes et al., 1987, Hörner et al., 1995
Fruit fly	Valles and White, 1988	Nässel and Elekes, 1992

are stimulated and initiates a tail flip that propels the crayfish away from the source of the stimulus (Wine, 1984). Initial experiments indicated that 5HT reduced the probability that the escape reflex was evoked by decreasing transmission between the sensory receptors and the lateral giant neurons (LGs)(Glanzman and Krasne, 1983); the amplitude of EPSPs evoked in the LGs decreased following the application of 5HT ( $10^{-3}$  to  $10^{-6}$  M). More recent studies, however, indicate that the modulation of this escape reaction is more complex, as 5HT has differing effects on the escape response in dominant and subordinate crayfish (Yeh et al., 1996); in dominant animals, 5HT actually enhances the escape reaction.

Another escape reaction modulated by 5HT is the cockroach escape response (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992a). Cockroaches escape from wind puffs by turning away from the source of wind and running. The escape response is evoked by the stimulation of wind-sensitive hair afferents on the cockroach cerci. The afferents connect with ventral giant interneurons (vGIs), which in turn connect with a population of approximately 100 identifiable thoracic interneurons (Casagrand and Ritzmann, 1992b). The thoracic interneurons in turn connect with leg motoneurons which mediate the escape response (Ritzmann and Pollack, 1990). Under certain conditions e.g. walking, this response can be modified (Camhi and Nolen, 1981), which implies the neural circuitry responsible for the behaviour is modulated. 5HT has been shown to reduce the response of the leg motoneurons to vGI stimulation (Goldstein and Camhi, 1991) and this occurs, at least in part, at the cholinergic synapses between the vGIs and thoracic interneurons (Casagrand and Ritzmann, 1992a, b); 5HT ( $10^{-4}$ - $10^{-3}$  M) significantly decreases the vGI-evoked EPSPs in the thoracic interneurons (Casagrand and Ritzmann, 1992a). In addition, 5HT also significantly

decreases cholinergic responses recorded from one of the leg motoneurons ( $D_f$ ) (Butt and Pitman, 1998), which suggests that 5HT also reduces transmission between the thoracic interneurons and leg motoneurons. As 5HT appears to inhibit transmission in this neuronal pathway, it indicates that 5HT would reduce the probability of evoking the escape response in the cockroach (Goldstein and Camhi, 1991) and may explain why the escape response is less likely to be evoked during certain behaviours.

Interestingly, dopamine has the opposite effect to 5HT on the neural circuit controlling the cockroach escape response (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992b); dopamine increases the response of the leg motoneurons to the activation of the vGIs (Goldstein and Camhi, 1991). This enhancement appears to occur at the cholinergic synapses between the vGIs and thoracic interneurons as application of dopamine ( $10^{-3}$  M) causes the amplitude of the vGI-evoked EPSPs recorded from the thoracic interneurons to increase by 20-50% (Casagrand and Ritzmann, 1992a).

However, dopamine also appears to have an antagonistic effect on the escape response pathway as it reduces the cholinergic responses recorded from one of the  $D_f$  motoneuron (Butt and Pitman, 1998), which suggests that dopamine inhibits transmission between the thoracic interneurons and leg motoneurons. The overall affect of dopamine on the neuronal pathway that controls the cockroach escape response does appear to be facilitatory (Goldstein and Camhi, 1991) and indicates the escape response is more likely to be evoked and may explain why the escape response is more likely to be evoked during certain behaviours e.g. walking (Camhi and Nolen, 1981).

5HT and dopamine have also been shown to have antagonistic effects on the neurons which control other behaviours. For example, the flight motor pattern in the mesothoracic ganglion of *Manduca sexta* is evoked by dopamine and the dopamine-

induced flight motor pattern is inhibited by 5HT (Claassen and Kammer, 1986). The dopamine-induced initiation of the flight motor pattern in *Manduca sexta* is site specific as injection of dopamine ( $1.5 \times 10^{-9}$  M) into the medial regions of the mesothoracic ganglion evokes the motor pattern but injection of dopamine ( $2.4 \times 10^{-8}$  M) into more lateral regions does not. The flight motor pattern evoked by dopamine is evoked in the presence or absence of flight motor input, suggesting dopamine acts on the neurons which generate the flight motor pattern. Dopamine also generates rhythmic motor activity in *Limax maximus* (Wieland and Gelperin, 1983). Application of dopamine to the isolated buccal ganglia of *Limax maximus* evokes rhythmic motor activity that resembles the feeding motor pattern (Wieland and Gelperin, 1983). 5HT has also been reported to generate a motor pattern. Bath application of 5HT to the isolated nerve cord of the medicinal leech initiates a swimming motor pattern (Willard, 1981).

As well as generating rhythmic motor activity, 5HT and dopamine have been reported to modulate the neuronal circuit, which produce the rhythmic motor patterns i.e. the central pattern generators. For example, the gastric and pyloric central motor pattern generators in the crustacean stomatogastric system, which control the muscles of the stomach, are modulated by 5HT and dopamine (Flamm and Harris-Warrick, 1986; Katz and Harris-Warrick, 1989, 1990). The cellular basis of this neuromodulation was examined by analysing the effects of the amines on the neurons in the gastric and pyloric neural circuits. Neurons in the gastric and pyloric neural circuits receive inputs from gastropyloric receptor (GPR) cells, which are serotonergic and cholinergic (Katz et al., 1989) and increase the excitability of the neurons in the gastric and pyloric neural circuits when stimulated. 5HT induces depolarising plateau potentials in the gastric neurons and initiates and increases rhythmic activity in the pyloric neurons (Katz and

Harris-Warrick, 1989, 1990). Dopamine has also been reported to modulate the neurons involved in the pyloric circuit. Dopamine depolarises and initiates or enhances action potentials in some neurons (lateral pyloric, pyloric and inferior cardiac neurons) but hyperpolarises and abolishes action potentials in others (ventral and pyloric dilator neurons) (Flamm and Harris-Warrick, 1986).

The studies described above reveal that 5HT and dopamine can modulate and generate rhythmic motor patterns and neural pathways that control motor behaviour e.g. escape reflexes. 5HT and dopamine have also been implicated in the modulation of learning and memory. The neural circuit which controls the gill and siphon withdrawal reflex in the mollusc *Aplysia californica* has been used as a model system to examine the cellular basis of learning, as the strength of the connections between the neurons involved in change in response to behavioural training (see Byrne and Kandel, 1996). Under normal conditions, the withdrawal reflex is evoked when the siphon is mechanically stimulated. After behavioural training it was found that this reflex was sensitised, i.e. more responsive, when a noxious stimulus was presented to the head or tail. This sensitisation is caused by the presynaptic facilitation of synaptic transmission between the siphon sensory neurons and motoneurons and interneurons in the reflex pathway (Castellucci and Kandel, 1976; Dale et al., 1988). The presynaptic facilitation of neurotransmitter release from the sensory neurons is mediated by 5HT via second messengers (Brunelli et al., 1976). 5HT causes an increase in the cAMP levels (Brunelli et al., 1976), which activates protein kinases (Castellucci et al., 1980) to phosphorylate a specific S (serotonin)-type  $K^+$  channel (Klein et al., 1982; Siegelbaum et al., 1982; Abrams et al., 1984). Closure of the S-type  $K^+$  channel by the cAMP-dependent protein kinases (Shuster et al., 1985), prolongs the action potentials in the

sensory neurons (Goldsmith and Abrams, 1992) and leads to an increase in  $\text{Ca}^{2+}$  influx (Klein and Kandel, 1980), which enhances transmitter release.

In contrast to the facilitatory effects 5HT has on learning in *Aplysia*, 5HT and dopamine appear to inhibit learning and memory in the honey bee, *Apis mellifera* (Mercer and Menzel, 1982; Mercer and Erber, 1983; Macmillan and Mercer, 1987; Bicker and Menzel, 1989). Associative learning is produced in honey bees by pairing a conditioned stimulus with an unconditioned stimulus. After a single conditioning trial, honey bees associate a conditioned stimulus (e.g. an odour) with an unconditioned stimulus (e.g. a sucrose reward) and respond to the odour with a response normally only caused by the sucrose reward i.e. proboscis extension. Injection of 5HT or dopamine into the brains of *Apis mellifera* (Mercer and Menzel, 1982) or application of dopamine to the antennal lobes of *Apis mellifera* (Macmillan and Mercer, 1987) results in a decrease in the percentage of bees which respond to the odour i.e. they do not associate the odour with a sucrose reward. The decrease in the response to the conditioned stimulus induced by the amines is independent of whether the amines are injected before or after conditioning and the responses to the conditioned stimulus increase and return to a level similar to that of the control groups, 60 minutes after amine injection. This indicates that 5HT and dopamine do not affect memory storage but do inhibit memory retrieval. The dopamine-induced decrease in the percentage of bees, which respond to a conditioned olfactory stimulus, may be accounted for by the effect of dopamine on neurons in the mushroom bodies of *Apis mellifera*. The amplitude of olfactory evoked potentials recorded from the  $\alpha$ -lobe of the mushroom bodies which processes olfactory information, decreases after dopamine is applied (Mercer and Erber, 1983).



These studies described above reveal that the biogenic amines modulate and generate certain behaviours in the invertebrate CNS, and the cellular basis of the modulation has been revealed by examining the effects of the amines on the neuronal pathways which control the behaviours. The receptors and the ionic mechanisms which are responsible for the effects the amines have on the neurons have been examined more extensively in a number of preparations.

5HT is reported to modulate a number of different ionic currents in different neurons (Siegelbaum et al., 1982; Bermudez, et al., 1992; Cuttle et al., 1995; Mercer et al., 1995). 5HT is reported to modulate the  $K^+$  conductance in *Aplysia* siphon sensory neurons (Siegelbaum et al., 1982), locust photoreceptor cells (Cuttle et al., 1995) and cultured antennal lobe neurons (Mercer et al., 1995). Certain inward currents evoked by 5HT in locust thoracic neurons are also due to a decrease  $K^+$  conductance and these are reported to be blocked by vertebrate 5HT<sub>2</sub> receptor antagonists (Bermudez, et al., 1992). Other inward currents evoked by 5HT in locust thoracic neurons are due to an increase in  $Na^+$  and are reported to be blocked by a vertebrate 5HT<sub>3</sub> receptor antagonist (Bermudez, et al., 1992).

Regardless of their pharmacological profiles, the majority of invertebrate 5HT receptors, like most vertebrate 5HT receptors, appear to be G-protein-coupled (Drummond et al., 1980; Berridge and Heslop, 1981; Cattell et al., 1985; Wedemeyer et al., 1992). Evidence of 5HT receptors coupled to adenylate cyclase have been reported in the cockroach (Nathanson and Greengard, 1973; Downer et al., 1985), blow fly (Berridge and Heslop, 1981), *Aplysia* (Brunelli et al., 1976; Castellucci et al., 1980; Drummond et al., 1980), fruit fly (Uzzan and Dudai, 1982) and locust (Orchard et al.,

1983; Cattell et al., 1985). Additionally, three 5HT receptors cloned from *Drosophila* also appear to be coupled to adenylate cyclase (Witz et al., 1990; Saudou et al., 1992). When expressed in mammalian cells, 5HT-dro1 receptors are positively coupled to adenylate cyclase, whereas 5HT-dro2A and 5HT-dro2B receptors inhibit adenylate cyclase activity. Activation of 5HT-dro2A and 5HT-dro2B receptors also activates phospholipase C (Saudou et al., 1992).

Evidence that invertebrate dopamine receptors are also coupled to adenylate cyclase has been revealed in studies that show the presence of dopamine-sensitive adenylate cyclases in the moth (Bodnaryk, 1979), cockroach (Nathanson and Greengard, 1973; Downer et al., 1985; Orr et al., 1987), fruit fly (Uzzan and Dudai, 1982) and mosquito (Pratt and Pryor, 1986). Some dopamine receptors on molluscan cells also appear to be coupled to adenylate cyclase and stimulate cAMP synthesis, as cAMP analogues mimic the effects of dopamine (Deterre et al., 1982; Stoof et al., 1984; de Vlieger et al., 1986). Some of these receptors mediate a hyperpolarising response in which  $K^+$  channels play a major role (de Vlieger et al., 1986) and some have pharmacological profiles partially consistent with that of vertebrate  $D_1$  receptors (Stoof et al., 1984). As well as dopamine receptors positively coupled to adenylate cyclase there is evidence that other dopamine receptors are negatively coupled to adenylate cyclase (Stoof et al., 1984).

Dopamine receptors on cockroach salivary gland acinar cells are positively coupled to adenylate cyclase, which is responsible for secretion from the cells (Gray et al., 1984) but there is evidence that they also activate a different second messenger pathway, coupled to an influx of  $Ca^{2+}$  ions (Evans and Green, 1990), that evokes the electrical response to dopamine (Ginsborg et al., 1980). Examination of the pharmacological

profile of the dopamine receptor, which mediates the responses in cockroach salivary gland cells, reveals a profile similar to that of the vertebrate D<sub>1</sub> dopamine receptor (Evans and Green, 1990).

The receptors, which mediate the effects of dopamine on the cockroach prothoracic inhibitory neuron (Pitman and Fleming, 1985; Pitman and Baker, 1989) and on the *Lymnaea* pedal giant and buccal neurons (Audesirk, 1989), do not appear to be coupled to adenylate cyclase. The dopamine responses are not significantly altered by an adenylate cyclase stimulant, the analogue dibutyryl cAMP or a phosphodiesterase inhibitor (Pitman and Fleming, 1985; Pitman and Baker, 1989; Audesirk, 1989).

Activation of the dopamine receptors on the cockroach prothoracic inhibitory neuron results in depolarising responses which involve Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup> and Cl<sup>-</sup> ions (Pitman and Davis, 1988) and have a novel effect on the activity of the neuron (Davis and Pitman, 1991). The response to dopamine is small when the membrane potential of the inhibitory neuron is at normal resting levels, but larger when the membrane potential is more depolarised (Davis and Pitman, 1991). This indicates that dopamine has a greater effect if the membrane is already depolarised, which could facilitate plateau potentials. Activation of the dopamine receptors on the *Lymnaea* pedal giant and buccal neurons evokes hyperpolarising responses that are, at least partly, due to an efflux of K<sup>+</sup> ions (Audesirk, 1989). The pharmacological profiles of these receptors and the dopamine receptors on the cockroach prothoracic inhibitory neuron are inconsistent with those of either vertebrate D<sub>1</sub> or D<sub>2</sub> receptors (Audesirk, 1989; Pitman and Davis, 1991).

The importance of the biogenic amines, 5HT and dopamine as neuromodulators in the invertebrate CNS is evident from the studies described above. It is possible that the

biogenic amines, 5HT and dopamine are also involved modulating the locust fSR/BA1 synapse. 5HT has already been reported to be capable of modulating the fSR peripherally, albeit through octopamine receptors (Ramirez and Orchard, 1990). In addition, the fSR, and cells to which it is presynaptic, are in synaptic or close contact with processes that contain large dense cored vesicles, which are morphologically similar to those in serotonergic and dopaminergic processes (see Chapter 4). Therefore, the aims of the experiments conducted in this chapter were; a) to establish if 5HT and dopamine do modulate cholinergic synaptic transmission between the fSR and BA1, b) to determine if 5HT and dopamine modulate the synapse by acting on the postsynaptic neuron BA1, and/or presynaptic neurons, c) to examine the possibility that the amines act at the same receptors as octopamine and d) to examine the potential sources of 5HT and dopamine.

## **6.2. MATERIALS AND METHODS**

### **6.2.1. Electrophysiology**

Electrophysiology was used to investigate the possibility that the amines, 5HT and dopamine modulate synaptic transmission between the fSR and BA1. The methods used to evoke synaptic transmission between the fSR and BA1 and elicit cholinergic responses in BA1 are described in detail in Chapter 2.2. The effects of 5HT and dopamine (Sigma, Poole, U.K.) on electrically-evoked EPSPs and cholinergic responses were tested.

The effects of dopamine and 5HT on the electrically-evoked EPSPs and ACh responses recorded from BA1 were calculated as percentage changes in control amplitude. All percentage means and standard errors of percentage means reported in the results have been arc-sine transformed unless otherwise stated. A two-tailed paired t-test was used to assess the significance of the percentage changes between control amplitudes and amplitudes in the presence of compounds. A two-tailed unpaired t-test was used to assess the significance of the percentage changes in response amplitudes between different experiments.

### **6.2.2. Confocal microscopical immunocytochemistry**

Confocal microscopical immunocytochemistry was used to determine if 5HT is present in the neuropil region where it could potentially modulate the fSR/BA1 synapse. The general method used to immunolabel transverse slices through the mesothoracic ganglia for confocal microscopy has been described in detail in Chapter 2.6. The method used to label the slices with an antibody to 5HT was modified slightly however as the antibody is conjugated to a different fixative.

Mesothoracic ganglia were fixed in 4% paraformaldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.4) containing 0.25% glutaraldehyde and 0.2 % picric acid. After being fixed and washed (see Chapter 2.6) the transverse slices through the mesothoracic ganglia were incubated overnight at 4°C in rabbit anti-5HT antiserum (Incstar, Stillwater, U.S.A.) was diluted 1:500 with Tris/BSA/X (pH 7.4). The anti-5HT antiserum was obtained from rabbits after immunisation with a 5HT-paraformaldehyde-BSA conjugate.

Positive and negative controls were performed to test the specificity of the labelling. The distribution of labelling in the slices incubated with the anti-5HT antibody was examined to determine if the antibody labelled known neuropil regions and tracts which are reported to be 5HT-IR in the locust metathoracic ganglion (Tyrrer et al., 1984). In negative control tests, the primary antibody was omitted and replaced with phosphate buffer. The negative controls did not show any immunolabelling.

### **6.3. RESULTS.**

#### **6.3.1. Effects of 5HT on electrically-evoked EPSPs recorded from BA1**

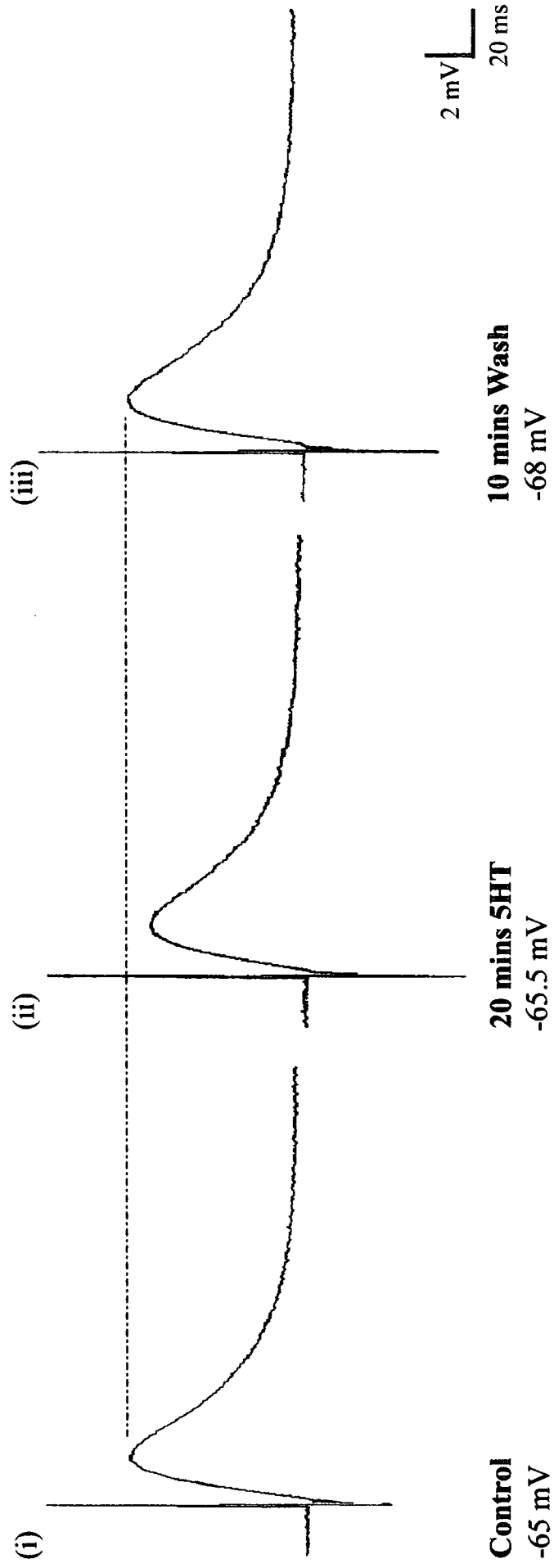
To investigate the possibility that the biogenic amine 5HT is potentially capable of modulating synaptic transmission between the fSR and BA1 the effects of 5HT ( $10^{-6}$  -  $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1 were examined.

Under control conditions, electrical stimulation of the fSR evoked EPSPs in BA1 with an amplitude of  $5.5 \pm 1.1$  mV (mean  $\pm$  SEM;  $n = 5$ ) and a duration of  $95.6 \pm 9.2$  ms (mean  $\pm$  SEM;  $n = 5$ ) (Figure 6.1i). Bath application of 5HT ( $10^{-4}$  M) resulted in a decrease in both the amplitude and duration of the EPSPs recorded from BA1 (Figure 6.1ii). In the majority of preparations the effects of 5HT on the EPSPs were only partially reversible after washing with drug-free saline, to 85% (+ SEM 4.2; - SEM 4.8;  $n = 4$ ) of the control EPSP amplitude, although the EPSP amplitude did reverse to control levels in one preparation (Figure 6.1iii).

**Figure 6.1. Effects of 5HT ( $10^{-4}$  M) on electrically-evoked EPSPs.**

(i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) 5HT ( $10^{-4}$  M) caused a decrease in the EPSP amplitude and duration. (iii) The effects of 5HT were reversible after washing with drug-free saline in one preparation (shown). In 4 preparations the effects of 5HT were partially reversible after washing (not shown). Each trace is an average of 8 successive electrically-evoked EPSPs. The membrane potential of BA1 at the time each group of 8 EPSPs was recorded is shown.

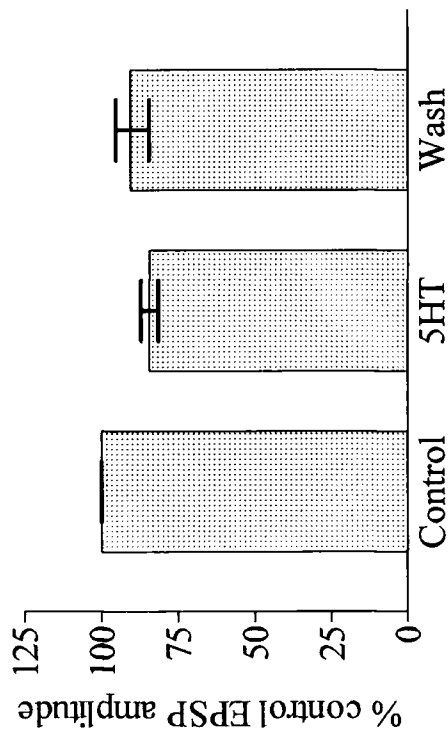
**Figure 6.1. Effects of 5HT ( $10^{-4}$  M) on electrically-evoked EPSPs.**



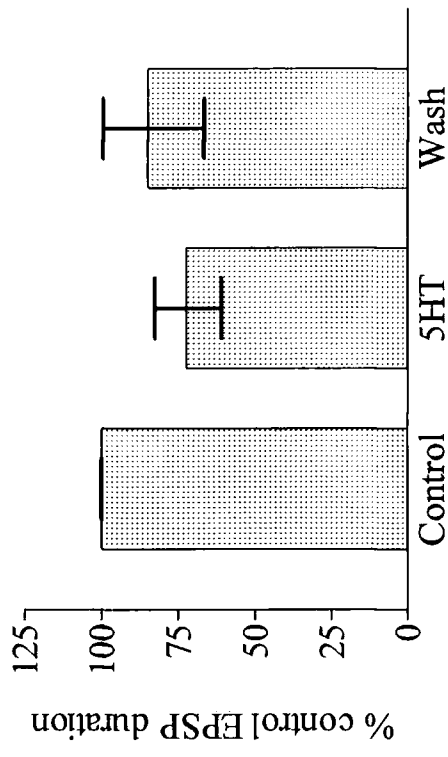


The EPSP amplitude began to decrease within 5 minutes of applying 5HT ( $10^{-4}$  M) and had decreased maximally within 20 minutes. The maximum mean decrease in the EPSP amplitude following 5HT application was 15.6 % (+ SEM 2.7; - SEM 2.9;  $p = 0.0005$ ;  $n = 5$ ) (Figure 6.2A) and the maximum mean decrease in EPSP duration was 27.6% (+ SEM 10.2; - SEM 11.5;  $p = 0.02$ ;  $n = 5$ ) (Figure 6.2B). However, the mean EPSP amplitude reversed to only 90.6 % (+ SEM 4.7; - SEM 6.1;  $p = 0.25$ ;  $n = 5$ ) of the control EPSP amplitude and the mean EPSP duration reversed to only 84.9% (+ SEM 14.6; - SEM 18.3;  $p = 0.1$ ;  $n = 5$ ) of the control EPSP duration, even after prolonged washing with drug-free saline.

To determine if the effects of 5HT ( $10^{-4}$  M) on the EPSPs were due to changes in the membrane conductance of the postsynaptic neuron BA1, the membrane potential and input resistance of BA1 were monitored. The input resistance of BA1 changed by only  $0.38 \pm 0.38 \text{ M}\Omega$  (mean  $\pm$  SEM) following the application of 5HT, which was statistically insignificant ( $p = 0.39$ ). In two preparations, the membrane potential of BA1 depolarised 1.5 - 3 mV following the application of 5HT. In one of these preparations, this resulted in BA1 depolarising to spiking threshold, which caused the electrical stimulation of the fSR to evoke action potentials rather than EPSPs in BA1. In four preparations, the membrane potential of BA1 hyperpolarised 0.5 - 6 mV following the application of 5HT. Although it is unclear why 5HT causes both depolarisations and hyperpolarisations in the same motoneuron in different preparations, it is possible that it is related to the initial control resting potentials of BA1. If the control resting potential of BA1 was relatively hyperpolarised (-65 - -67.5 mV), application of 5HT caused BA1 to depolarise (Figure 6.1) whereas, if the control resting potential of BA1 was relatively depolarised (-57 to -65 mV), application of 5HT



**Figure 6.2A. Maximum effect of 5HT ( $10^{-4}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of 5HT and following wash ( $n = 5$ ). The amplitude is given as a percentage of the control amplitude.



**Figure 6.2B. Maximum effect of 5HT ( $10^{-4}$  M) on EPSP duration.** Graphical representation of the pooled data from 5 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of 5HT and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.

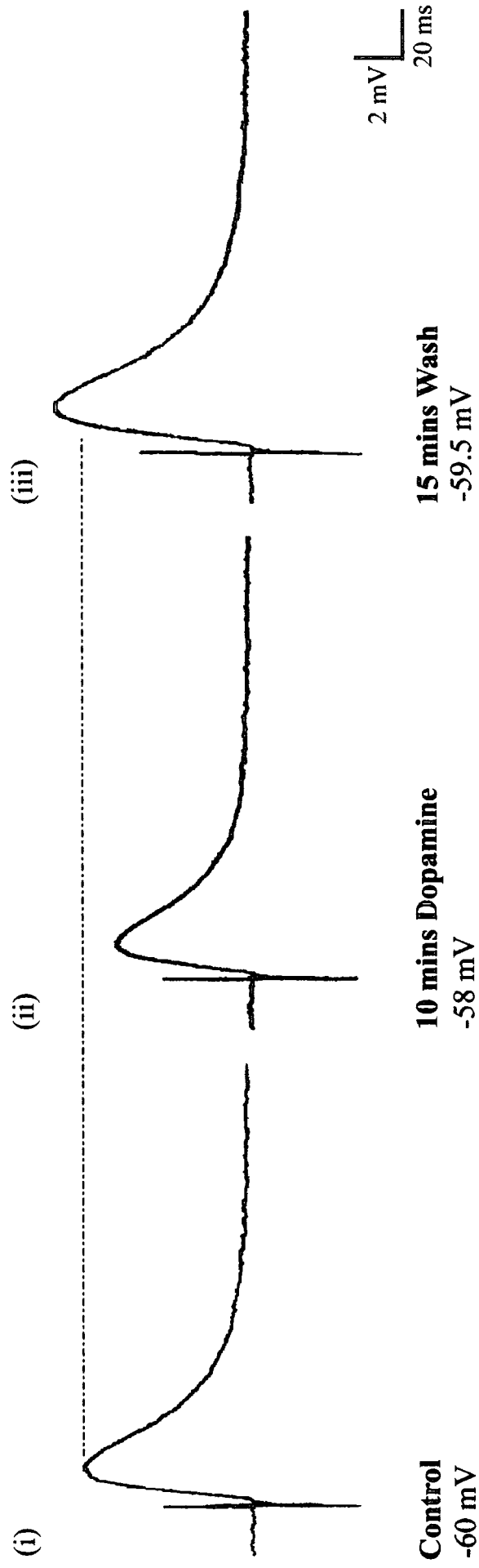
caused BA1 to hyperpolarise. Therefore, 5HT generally caused the membrane potential of BA1 to shift to approximately -65 mV. Although 5HT did cause a change in the membrane potential the change in potential does not appear to be responsible for the decrease in the EPSP amplitude and duration. The EPSP amplitude was suppressed even when BA1 repolarised in the presence of 5HT, which indicates that mechanisms other than changes in the membrane conductance of BA1 are involved in the modulation of the EPSP.

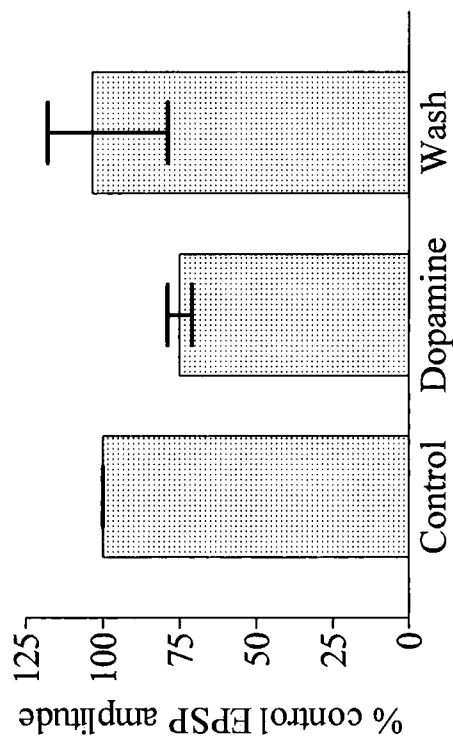
Different concentrations of 5HT have been reported to have opposite modulatory effects on synaptic transmission (Kloppenborg and Hildebrand, 1995). For example, 5HT ( $10^{-4}$  M) enhances the responses of *Manduca sexta* antennal-lobe neurons to the electrical stimulation of the antennal nerve, whereas 5HT ( $10^{-8}$  M) suppresses the responses (Kloppenborg and Hildebrand, 1995). The effects of lower concentrations of 5HT on electrically-evoked EPSPs recorded from BA1 were, therefore, examined to determine if different concentrations of 5HT had opposite modulatory effects on synaptic transmission between the fSR and BA1.

Bath application of 5HT ( $10^{-5}$  M) caused the EPSP amplitude to decrease by 10 %. The EPSP amplitude only began to decrease 10 minutes after the application of 5HT ( $10^{-5}$  M), which was accompanied by the membrane potential of BA1 hyperpolarising 1 mV. The effects of 5HT ( $10^{-5}$  M) on the EPSP amplitude reversed after washing the preparation with drug-free saline. Bath application of 5HT ( $10^{-6}$  M) had no effect on the EPSP amplitude or the membrane potential of BA1. It appears therefore, that the only modulatory effect 5HT has on synaptic transmission between the fSR and BA1 is to suppress it in a concentration dependent manner.

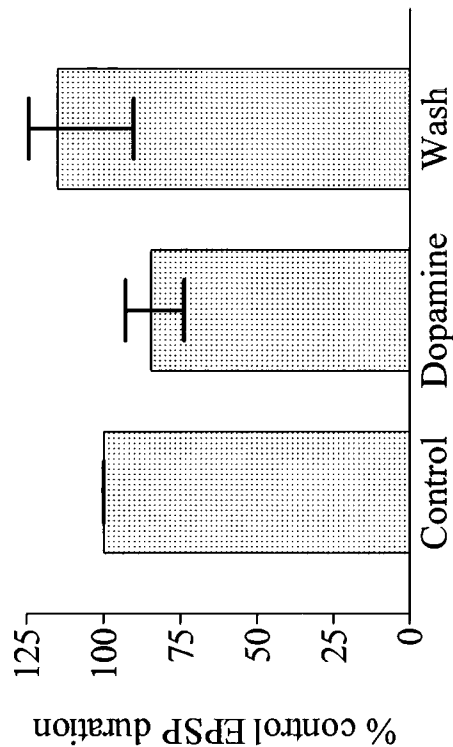
**Figure 6.3. Effects of Dopamine ( $10^{-4}$  M) on electrically-evoked EPSPs.** (i) Excitatory postsynaptic potentials (EPSPs) were evoked in BA1 by the electrical stimulation of the fSR axon. (ii) Dopamine ( $10^{-4}$  M) caused a decrease in the EPSP amplitude and duration. (iii) The effects of dopamine were reversible after washing with drug-free saline. Each trace is an average of 8 successive electrically-evoked EPSPs. The membrane potential of BA1 at the time each group of 8 EPSPs was recorded is shown

**Figure 6.3. Effects of Dopamine ( $10^{-4}$  M) on electrically-evoked EPSPs.**





**Figure 6.4A. Maximum effect of Dopamine ( $10^{-4}$  M) on EPSP amplitude.** Histogram showing the mean ( $\pm$  SEM) amplitude of EPSPs recorded from BA1 in the absence (Control) and presence of dopamine and following wash ( $n = 2$ ). The amplitude is given as a percentage of the control amplitude.



**Figure 6.4B. Maximum effect of Dopamine ( $10^{-4}$  M) on EPSP duration.** Graphical representation of the pooled data from 2 experiments showing the duration of the EPSPs recorded from BA1 in the absence (Control) and presence of dopamine and following wash. The mean ( $\pm$  SEM) duration is given as a percentage of the control duration.

### 6.3.2. Effects of dopamine on electrically-evoked EPSPs recorded from BA1

To investigate the possibility that the biogenic amine dopamine is also potentially capable of modulating synaptic transmission between the fSR and BA1 the effects of dopamine ( $10^{-4}$  M) on electrically-evoked EPSPs recorded from BA1 were examined.

Under control conditions, electrical stimulation of the fSR evoked EPSPs in BA1 with a mean amplitude of  $5.6 \pm 0.3$  (mean  $\pm$  SEM;  $n = 3$ ) and a duration of  $112.3 \pm 26$  (mean  $\pm$  SEM;  $n = 3$ ) (Figure 6.3i). Following the bath application of dopamine ( $10^{-4}$  M) both the amplitude and duration of the EPSP recorded from BA1 decreased (Figure 6.3ii). The effects of dopamine ( $10^{-4}$  M) on the EPSP reversed to control levels after washing with drug-free saline (Figure 6.3iii). The EPSP amplitude decreased by a mean percentage of 24.9 % (+ SEM 3.5; - SEM 4.1;  $p = 0.056$ ;  $n = 2$ ) (Figure 6.4A) and the duration by a mean percentage of 15.3 % (+ SEM 10.7; - SEM 8.3;  $p = 0.02$ ;  $n = 2$ ) (Figure 6.4B). Washing with drug-free saline caused the mean EPSP amplitude and duration to return to control levels (Figure 6.4).

However, the decrease in EPSP amplitude and duration could not be observed until approximately 5 minutes after the application of dopamine. This was because within 2 minutes of applying dopamine the membrane potential of BA1 depolarised (3 - 14 mV) to spiking threshold ( $n = 3$ ). This resulted in spontaneous action potentials and in electrically driven EPSPs being sufficient to evoke action potentials rather than EPSPs in BA1. The membrane potential of BA1 began to repolarise (1 - 4 mV) 5 minutes after

dopamine application. This allowed EPSPs to be recorded from BA1 in only two preparations.

As the membrane potential of BA1 did not repolarise to the control resting potential in the presence of dopamine, the decrease in the EPSP amplitude and duration could possibly be a consequence of the change in the membrane potential of BA1. However, the difference between the control membrane potential and the membrane potential in the presence of dopamine (2 - 3 mV) is unlikely to be sufficient to account for the marked decrease in the EPSP amplitude, indicating that another mechanism may be involved. In addition, the input resistance of BA1 only changed by  $0.8 \pm 1.4 \text{ M}\Omega$  (mean  $\pm$  SEM;  $p = 0.62$ ) following the application of dopamine. These observations indicate that a change in the membrane conductance of BA1 seems unlikely to be the cause of the decrease in the EPSP amplitude and duration.

To summarise, the decrease in the EPSP amplitude and duration following the application of the biogenic amines 5HT and dopamine indicates that 5HT and dopamine are potentially capable of inhibiting cholinergic transmission between the fSR and BA1. The mechanisms, which mediate the inhibitory actions of the biogenic amines are undetermined, although the results do indicate that mechanisms other than changes in the membrane conductance of BA1 are involved.

### **6.3.3. Effects of 5HT on the responses of BA1 to locally-applied ACh**

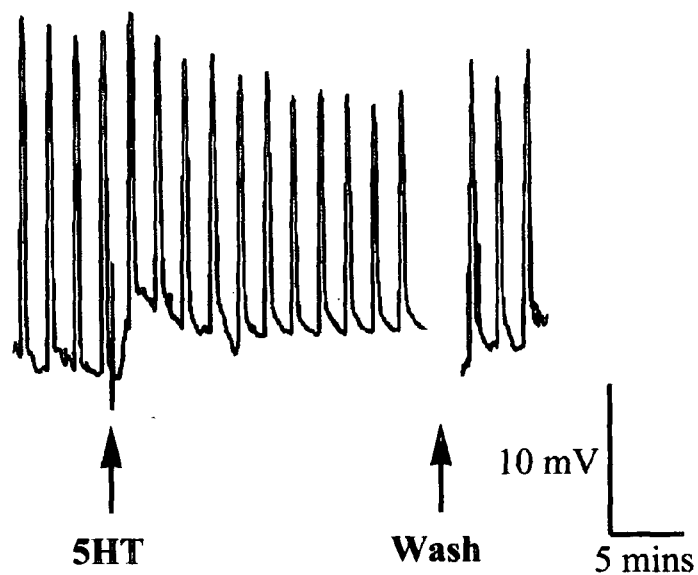
The biogenic amine 5HT may suppress synaptic transmission between the fSR and BA1 by inhibiting ACh release from the presynaptic terminals of the fSR and/or by



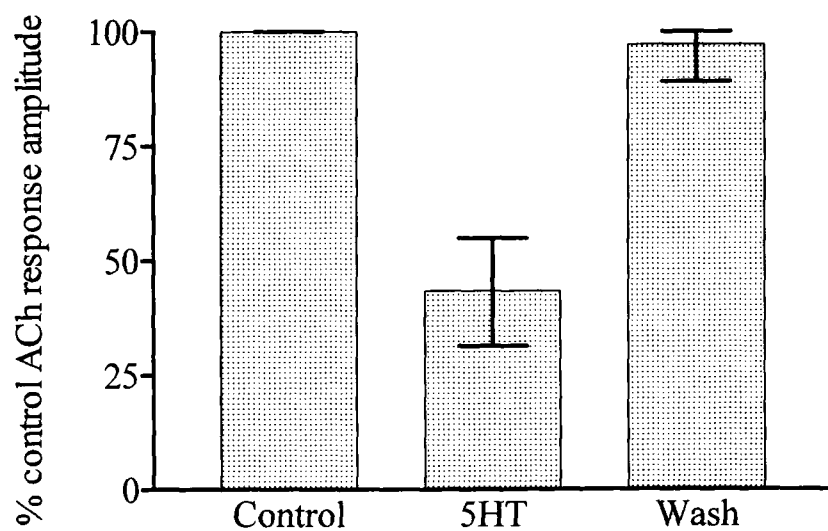
**Figure 6.5A. Effects of 5HT ( $10^{-4}$  M) on ACh responses recorded from BA1.** Brief pulses (50 - 200 ms) of acetylcholine (ACh), directly applied to the cell body of BA1, evoked depolarising responses in BA1. Application of 5HT ( $10^{-4}$  M) caused a decrease in the amplitude of the ACh response. The decrease in ACh response amplitude reversed partially after washing.

**Figure 6.5B. Maximum effect of 5HT ( $10^{-4}$  M) on ACh response amplitude.** Graphical representation of the pooled data from 3 experiments showing the amplitude of the ACh responses recorded from BA1 in the absence (control) and presence of 5HT and following wash. The mean ( $\pm$  SEM) amplitude is given as a percentage of the control amplitude.

**Figure 6.5A. Effects of 5HT ( $10^{-4}$  M) on ACh responses recorded from BA1.**



**Figure 6.5B. Maximum effect of 5HT ( $10^{-4}$  M) on ACh response amplitude.**



reducing the response of BA1 to ACh. To distinguish between these possibilities, the effects of 5HT ( $10^{-4}$  M) on the response of BA1 to locally-applied ACh were examined.

Under control conditions pressure application of brief (50 - 200 ms) pulses of ACh (100 mM) to the cell body of BA1 resulted in depolarising responses with a mean amplitude of  $19 \pm 2.1$  mV (mean  $\pm$  SEM;  $n = 3$ ). Following the bath application of 5HT ( $10^{-4}$  M) the amplitude of the ACh response began to decrease within 2 minutes and had decreased maximally within 15 minutes (Figure 6.5A). The ACh response amplitude decreased by a maximum mean percentage of 56.6 % (+ SEM 11.5; - SEM 12.1;  $p = 0.02$ ;  $n = 3$ ) and partially reversed after washing with drug-free saline (Figure 6.5B).

The membrane potential and input resistance of BA1 were monitored throughout the experiments to determine if the effects of 5HT on the ACh responses of BA1 were due to changes in the membrane conductance of BA1. The input resistance of BA1 did not change ( $p = 0.5$ ) following the application of 5HT but the membrane potential did. In one preparation the membrane potential of BA1 hyperpolarised by 7 mV and in two preparations the membrane potential of BA1 depolarised 4 - 9.5 mV. However, the changes in the membrane potential of BA1 do not appear to be responsible for the decrease in the ACh response amplitude following the application of 5HT, as the ACh response amplitude was suppressed, even when the membrane potential of BA1 repolarised ( $n = 2$ ). This indicates that mechanisms other than changes in the membrane conductance of BA1 are involved in mediating the inhibitory effects of 5HT on the responses of BA1 to ACh.

#### 6.3.4. Effects of dopamine on the responses of BA1 to locally-applied ACh

To investigate the possibility that the biogenic amine dopamine suppresses the response of BA1 to ACh, the effects of dopamine ( $10^{-4}$  M) on the response of BA1 to locally-applied ACh were examined.

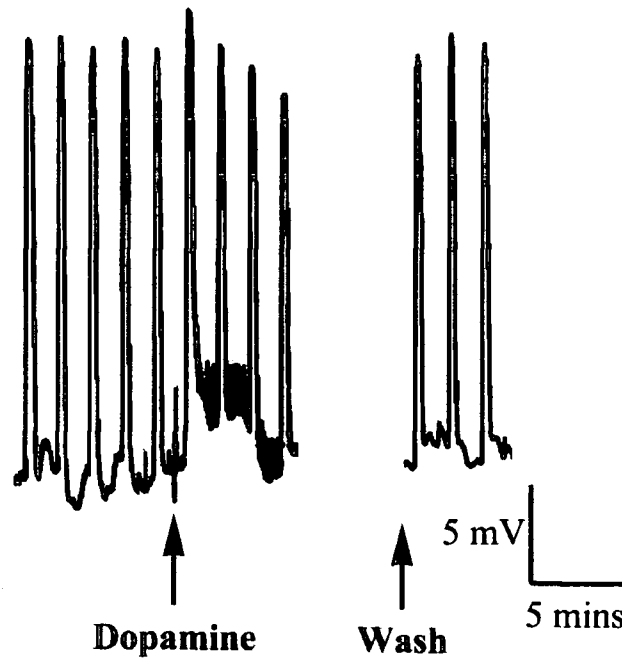
Under control conditions pressure application of brief (50 - 200 ms) pulses of ACh (100 mM) to the cell body of BA1 resulted in depolarising responses with a mean amplitude of  $20 \pm 2.2$  mV (mean  $\pm$  SEM;  $n = 5$ ). Following the bath application of dopamine ( $10^{-4}$  M) the amplitude of the ACh response decreased (Figure 6.6A). The amplitude of the ACh response generally began to decrease within 2 minutes following the application of dopamine ( $10^{-4}$  M) and had reached a maximum decrease within 15 minutes following application. Bath application of dopamine ( $10^{-4}$  M) caused the ACh response amplitude to decrease by a maximum mean percentage of 13.7 % (+ SEM 4.1; - SEM 4.6;  $p = 0.0095$ ;  $n = 5$ ) (Figure 6.6B). Washing the preparation with drug-free saline caused the ACh response amplitude to return to control levels within 10 minutes.

To determine if the decrease in ACh response amplitude following the application of dopamine was due to a change in the membrane conductance of BA1, both the membrane potential and the input resistance of BA1 were monitored throughout the experiments. The membrane potential of BA1 depolarised 1 - 4.5 mV within 1 minute following the application of dopamine and then began to repolarise. However, approximately 5 minutes after the application of dopamine the membrane potential of BA1 began to depolarise again by 1 - 3.5 mV (see Figure 6.6A). Although the membrane potential of BA1 did fluctuate following dopamine application, the decrease

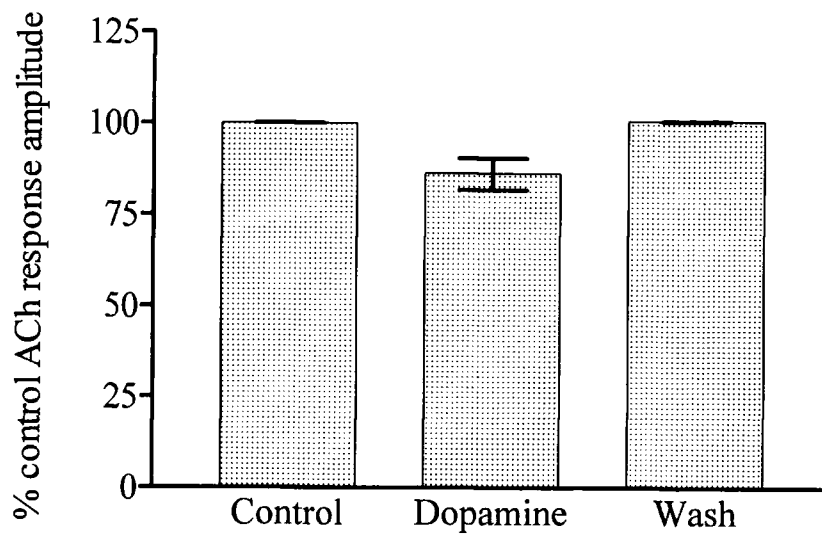
**Figure 6.6A. Effects of Dopamine ( $10^{-4}$  M) on ACh responses recorded from BA1.** Pressure-application of brief pulses (50-200 ms) of acetylcholine (ACh) onto the cell body of BA1, resulted in depolarising responses in BA1. Following the application of dopamine ( $10^{-4}$  M) the amplitude of the ACh responses decreased. The amplitude of the ACh responses returned to control levels after washing. Note that spontaneous activity is recorded from BA1 following the application of dopamine.

**Figure 6.6B. Maximum effect of Dopamine ( $10^{-4}$  M) on ACh response amplitude.** Graphical representation of the pooled data from 5 experiments showing the amplitude of the ACh responses recorded from BA1 in the absence (control) and presence of dopamine and following wash. The mean ( $\pm$  SEM) amplitude is given as a percentage of the control amplitude.

**Figure 6.6A. Effects of Dopamine ( $10^{-4}$  M) on ACh responses recorded from BA1.**



**Figure 6.6B. Maximum effect of Dopamine ( $10^{-4}$  M) on ACh response amplitude**



in ACh response amplitude does not appear to be a consequence of this. The amplitude of the ACh response remained consistently depressed even when the membrane potential repolarised, indicating that the decrease in the ACh response amplitude following dopamine application was not due entirely to a change in the membrane potential of BA1. In addition, the input resistance of BA1 did not change significantly ( $p = 0.25$ ) following the application of dopamine, indicating that a change in the membrane conductance of BA1 seems unlikely to be the cause of the decrease in the ACh response amplitude.

An additional observation during these experiments was the effect of dopamine had on spontaneous activity. In one preparation spontaneous activity was recorded from BA1 under control conditions. This appeared on the recordings as rapid membrane fluctuations with amplitudes of 0.5 -1 mV and was caused by other neurons, which make synaptic contacts with BA1. The spontaneous activity was inhibited within 1 minute following the application of dopamine, indicating dopamine inhibits neurons presynaptic to BA1. In two other preparations, no spontaneous activity was recorded from BA1 under control conditions, but following the application of dopamine, spontaneous activity appeared (see Figure 6.6A). This indicates that dopamine may also excite neurons presynaptic to BA1. After washing the preparation with drug-free saline spontaneous activity was no longer recorded from BA1.

To summarise, the biogenic amines 5HT and dopamine are capable of suppressing the response of BA1 to ACh. The mechanisms, which mediate the inhibitory actions of the biogenic amines on the ACh responses are undetermined, although the results do suggest that mechanisms other than changes in the membrane conductance of BA1,

such as receptor activation, are involved. The aminergic inhibition of synaptic transmission at the fSR/BA1 synapse therefore, appears to be due to the effects of the biogenic amines on the postsynaptic neuron although the significant difference in the effects of 5HT on ACh responses and EPSPs also suggests 5HT may affect presynaptic neurons.

### **6.3.5. Effect of 5HT on ACh responses in the presence of octopamine**

It is possible that the biogenic amines 5HT and dopamine are modulating the cholinergic response of BA1 not through 5HT or dopamine receptors but through a receptor for another amine. The results in Chapter 5 reveal that the biogenic amine octopamine is also capable of modulating the cholinergic response of BA1; bath application of DL-octopamine ( $10^{-4}$  M) caused the ACh response amplitude to decrease by 23% (+SEM 3.9; - SEM 1.2;  $p < 0.0001$ ;  $n = 10$ ). There is also strong evidence from studies on a number of insect preparations that 5HT and the biogenic amine octopamine act at a common receptor (Evans, 1981; Claassen and Kammer, 1986; Whim and Evans, 1988; Ramirez and Orchard, 1990). It is possible therefore, that 5HT and octopamine are acting at a common receptor or mediate their effects on the ACh responses through the same second messenger pathway. To investigate this possibility, the effect of 5HT ( $10^{-4}$  M) on the ACh response in the presence of octopamine ( $10^{-4}$  M), was examined. If 5HT and octopamine act at separate receptors on BA1 or activate separate second messenger pathways, application of 5HT in the presence of octopamine should cause a further decrease in the ACh response in addition to the decrease caused by octopamine i.e. their effects would be additive. If 5HT and octopamine share a common receptor and/or second messenger pathway, application of 5HT in the



presence of octopamine should not cause a further decrease in the ACh response amplitude, as the action of one amine should occlude that of the other.

Pre-incubating the preparation with DL-octopamine ( $10^{-4}$  M) resulted in the ACh response amplitude decreasing by 19.4% (+SEM 2.4; -SEM 2.6;  $p = 0.577$ ;  $n = 2$ ).

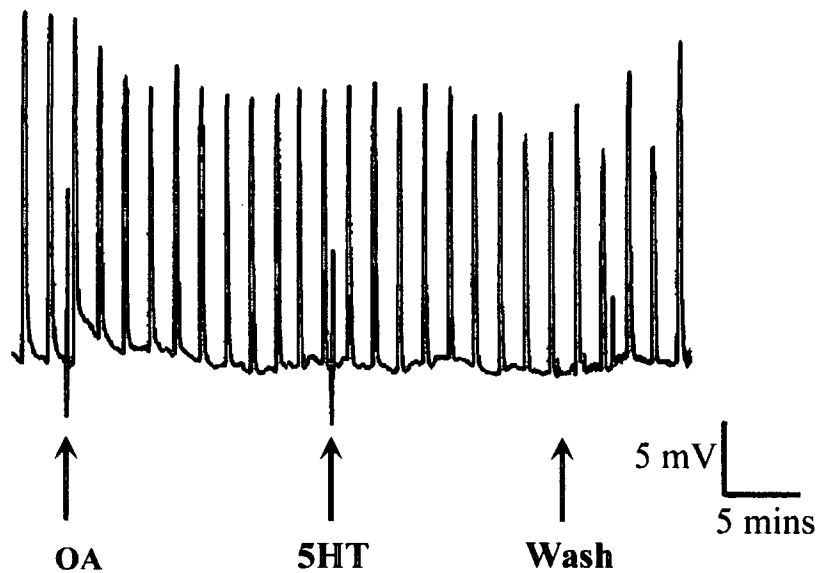
Once a stable set of ACh responses had been recorded with similar amplitudes in the presence of DL-octopamine, 5HT ( $10^{-4}$  M) was applied to the same preparation (Figure 6.7A). Although, the ACh response amplitude did decrease by an additional 8.5% (+SEM 4.9; -SEM 5.2;  $n = 2$ ) 5 minutes after 5HT application, the decrease was insignificant ( $p = 0.0724$ ) (Figure 6.7 B). The ACh response amplitude partially returned to control levels after washing the preparation with drug-free saline.

These experiments demonstrate that 5HT does not cause a significant decrease in the ACh response amplitude in the presence of octopamine, which indicates that pre-incubating the preparation with octopamine occludes the action of 5HT on the ACh response. These results are therefore consistent with the possibility that 5HT and octopamine do act at a common receptor on BA1. However, further neuropharmacological experiments are needed to determine if octopamine acts at a 5HT receptor or 5HT acts at an octopamine receptor to modulate the cholinergic response of BA1. It is worth mentioning though, that in other neuropharmacological studies on insect preparations that have reported similar findings, evidence is provided that indicates that the common receptors are octopamine receptors rather than 5HT receptors (Evans, 1981; Ramirez and Orchard, 1990).

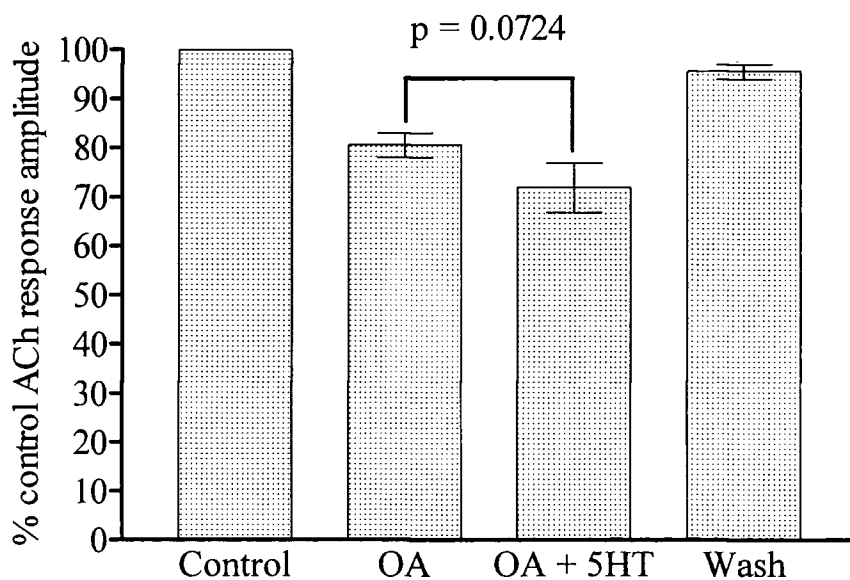
**Figure 6.7A. Effect of 5HT ( $10^{-4}$  M) on ACh responses recorded from BA1 in the presence of Octopamine ( $10^{-4}$  M) .** Pressure-application of brief pulses of acetylcholine (ACh) onto the cell body of BA1, resulted in depolarising responses in BA1. Following the application of DL-octopamine ( $10^{-4}$  M) the amplitude of the ACh responses decreased. Sequential application of 5HT ( $10^{-4}$  M) in the presence of octopamine caused only a slight decrease in the ACh response amplitude. The amplitude of the ACh responses partially returned to control levels after washing.

**Figure 6.7B. Effect of 5HT ( $10^{-4}$  M) on ACh response amplitudes in the presence of Octopamine ( $10^{-4}$  M).** Graphical representation of the pooled arc sine transformed data showing the mean ( $\pm$  SEM) amplitude of the ACh responses recorded from BA1 ( $n = 2$ ). The amplitude is given as a percentage of the control amplitude. The ACh response amplitude did not decrease significantly following the application of 5HT, in the presence of octopamine. The P value was calculated using a two-tailed t-test.

**Figure 6.7A. Effect of 5HT ( $10^{-4}$  M) on ACh responses recorded from BA1 in the presence of Octopamine ( $10^{-4}$  M).**



**Figure 6.7B. Effect of 5HT ( $10^{-4}$  M) on ACh response amplitudes in the presence of Octopamine ( $10^{-4}$  M).**



### 6.3.6. 5HT-immunoreactivity in aLAC

The results from the electrophysiological experiments demonstrate that 5HT and dopamine are potentially capable of modulating cholinergic transmission between the fSR and BA1, which is due (at least in part) to the amines suppressing the response of BA1 to ACh. This however, does not confirm that the amines modulate the fSR/BA1 synapse *in vivo*. The amines would have to be at least present in the dorso-lateral neuropil region that contains the fSR/BA1 synapses, to modulate synaptic transmission between the fSR and BA1. A previous study (Watson, 1992b) has shown that dopamine-like IR is present in the dorso-lateral neuropil regions of the locust mesothoracic ganglion indicating it could modulate the fSR/BA1 synapse. The distribution of 5HT-IR in the locust mesothoracic ganglion, however, has not been examined. To determine if 5HT is present in aLAC, the neuropil region into which the dendrites of the fSR and the flight motoneurons project, confocal microscopical ICC was carried out.

Transverse sections through the mesothoracic ganglion, which had been incubated with an anti-5HT antibody and labelled with Rhodamine Red, were examined under an inverted Nikon Axiophot microscope equipped with a Biorad Microradiance confocal system. Primary antibody omission controls were also inspected. Examination of the slices, which had been incubated with the anti-5HT antibody, revealed specific regions of red fluorescence, which indicated 5HT-IR. Primary antibody omission controls showed no red fluorescence. The level at which each slice had been taken through the mesothoracic ganglion was identified under light microscopical conditions using anatomical guides (Tyrer and Gregory, 1982; Pflüger et al., 1988). Therefore, the

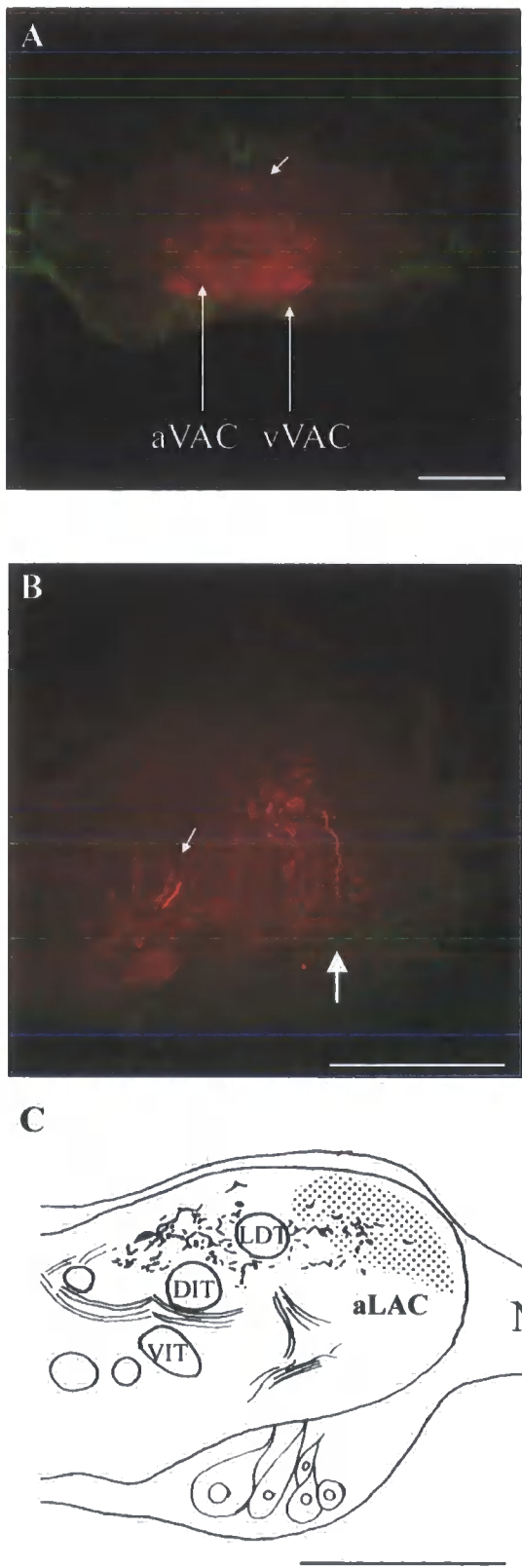
transverse sections which contained aLAC could be identified. Camera lucida drawings of the levels through the mesothoracic ganglion containing this neuropil region are shown in Figure 4.1.

The transverse slices taken at the level of nerve 3 (N3) contain aLAC and are shown in Figure 6.8. Examination of these slices revealed that the 5HT-IR represented by red fluorescence, was most dense in the ventral association centres (VAC). The 5HT-IR was most concentrated in the ventralmost VAC (vVAC) and along the dorsal edge of the anterior VAC (aVAC) (Figure 6.8A). Concentrated 5HT-IR was also found in the lateral VAC (lVAC) (Figure 6.8A). Individual branches that were 5HT-IR were found extending through the lVAC (Figure 6.8B), and also within the dorsal median tract (DMT) (Figure 6.8A) and the ventral intermediate tract (VIT) (Figure 6.8B). Cell bodies that were present in the slices containing aLAC were not 5HT-IR. Interestingly, the dorso-lateral neuropil regions, which contain aLAC, showed very little 5HT-IR (Figures 6.8A,B). Comparison of the confocal images of the 5HT-IR distribution and the camera lucida drawing showing the distribution of the fSR branches in Figure 6.8C, reveals that aLAC and the branches of the fSR are in an area of neuropil which is not 5HT-IR. This would seem to indicate that 5HT is not present in high concentrations in the neuropil region where it could potentially modulate the fSR/BA1 synapse.

**Figure 6.8. 5HT-immunoreactivity in the mesothoracic ganglion.**

Transverse sections taken through the mesothoracic ganglion at the level of nerve 3 (N3). (A, B) Confocal microscope images of a transverse slice incubated with an anti-5HT antibody and labelled with Rhodamine Red. 5HT-immunoreactivity (5HT-IR) is indicated by the red fluorescence. (A) 5HT-IR is most dense in the ventral association centres (VAC). The ventralmost VAC (vVAC) and the anterior VAC (aVAC) are labelled. 5HT-IR is also present in the paired median dorsal tracts (MDT) indicated by a small white arrow. (B) Confocal microscope image of the slice shown in (A) at a higher magnification and at a different depth. Note the individual 5HT-IR branches which can be seen in the lateral VAC (lVAC) indicated by the large white arrow. 5HT-IR axons can also be seen in the ventral intermediate tract (VIT) indicated by the small white arrow. (C) Camera lucida drawing showing the distribution of the fSR branches and the position of the anterior lateral association centre (aLAC) (stippled area) in the dorsal region of mesothoracic ganglion. Dorsal is to the top, lateral is to the right. DIT = dorsal intermediate tract, LDT = lateral intermediate tract. Scale bars = 200  $\mu$ m.

**Figure 6.8. 5HT-immunoreactivity in the mesothoracic ganglion.**



## 6.4. DISCUSSION

### 6.4.1. Aminergic modulation of cholinergic transmission

The results from the electrophysiological experiments presented in this chapter clearly demonstrate that both 5HT and dopamine are capable of modulating cholinergic synaptic transmission between the sensory afferent, fSR and the motoneuron, BA1; the amplitude of fSR-evoked EPSPs recorded from BA1 decreased in the presence of 5HT and dopamine. 5HT and dopamine could potentially modulate cholinergic synaptic transmission by regulating ACh release from the presynaptic terminals and/or modulating the responses of the postsynaptic neuron to ACh.

A classic example of aminergic modulation of ACh release from presynaptic terminals is the regulation of ACh release from the siphon sensory neurons in *Aplysia californica* by 5HT (Castellucci and Kandel, 1976; Dale et al., 1988). 5HT facilitates ACh release from the siphon sensory neurons, thus enhancing transmission between the neurons in the gill and siphon withdrawal reflex. Indirect evidence for the involvement of 5HT and dopamine in the modulation of ACh release has come from a number of studies which have reported that cholinergic neurons are modulated by the biogenic amines (Flamm and Harris-Warrick, 1986; Ramirez and Orchard, 1990). 5HT has been shown to increase the number and frequency of action potentials in the locust sensory afferent fSR (which had been shown to be cholinergic [Leitch and Pitman, 1995] ) in response to wing movements (Ramirez and Orchard, 1990). This increase in activity in the fSR caused by 5HT may in turn increase the release of ACh from the presynaptic terminals. The cholinergic neurons in the pyloric circuit of the lobster stomatogastric ganglion



(Marder, 1974, 1976) are also modulated by 5HT and dopamine (Flamm and Harris-Warrick, 1986). Dopamine inhibits action potentials in both the ventral dilator (VD) and the pyloric dilator (PD) neurons, whereas 5HT inhibits action potentials in the ventral dilator neuron but has no significant effect on the pyloric dilator neuron. These biogenic amines, therefore, may indirectly inhibit ACh release from these cholinergic neurons. There is also evidence from studies on vertebrate preparations that 5HT and dopamine may modulate ACh release from cholinergic neurons (Day and Fibiger, 1993, 1994; Khateb et al., 1993; Zarborsky and Cullinan, 1996; Smiley et al., 1999).

Microscopical studies have revealed that cholinergic neurons in the guinea-pig and primate basal forebrain are in close contact with 5HT-IR axons (Khateb et al., 1993; Smiley et al., 1999), and that cholinergic neurons in the rat and primate basal forebrain receive synaptic inputs from dopaminergic axons (Zarborsky and Cullinan, 1996; Smiley et al., 1999). The microscopical evidence that amines modulate cholinergic neurons in vertebrates is also supported by electrophysiological studies (Day and Fibiger, 1993, 1994; Khateb et al., 1993). The membrane potential of cholinergic neurons from the guinea-pig basal forebrain hyperpolarises following the application of 5HT (Khateb et al., 1993). This hyperpolarisation inhibits spontaneous firing and the rebound responses to hyperpolarising current injection. It also reduces the frequency of the responses to depolarising current injection. These results thus indicate that 5HT may potentially reduce ACh release from the cholinergic neurons in the basal forebrain. Dopamine, on the other hand, is reported to increase ACh release from neurons in the basal forebrain and hippocampus (Day and Fibiger, 1993, 1994).

Another way in which the amines could potentially modulate ACh release from the presynaptic terminals is by regulating choline uptake. As choline uptake can limit the

rate ACh is synthesised, aminergic modulation of choline uptake can potentially regulate ACh release from the presynaptic terminals. The high affinity choline uptake system in the horseshoe crab, *Limulus polyphemus* has been reported to be modulated by 5HT (Ford et al., 1995). Application of 5HT ( $10^{-5}$  M) increased choline uptake by 41%, which may facilitate ACh release. This study and the others mentioned above provide evidence as to how cholinergic synaptic transmission could potentially be modulated by 5HT and dopamine regulating ACh release from the presynaptic terminals.

5HT and dopamine could also potentially modulate cholinergic transmission by modulating the responses of the postsynaptic neuron to ACh. The cholinergic responses of the VD and PD neurons, which are part of the pyloric circuit in the lobster stomatogastric ganglion, are modulated by 5HT and dopamine (Johnson and Harris-Warrick, 1997). Although 5HT ( $10^{-5}$  M) has no effect on the amplitude of the cholinergic responses recorded from the PD, the cholinergic responses recorded from the VD decrease by 34% following the application of 5HT ( $10^{-5}$  M) (Johnson and Harris-Warrick, 1997). Application of dopamine ( $10^{-4}$  M) on the other hand causes the amplitude of the cholinergic responses to decrease in both the PD and VD, by 39% and 38%, respectively (Johnson and Harris-Warrick, 1997). 5HT and dopamine have a similar effects on the cholinergic responses of the  $D_f$  motoneuron, which is involved in the escape pathway in the cockroach, *Periplaneta americana* (Butt and Pitman, 1998). Application of 5HT ( $10^{-4}$  M) causes a decrease of 50.4 % in the amplitude of the nicotinic cholinergic responses recorded from  $D_f$  and dopamine ( $10^{-4}$  M) causes a decrease of 22.6 % (Butt and Pitman, 1998).

Evidence that 5HT and dopamine reduce cholinergic synaptic transmission at the locust fSR/BA1 synapse by modulating the responses of the postsynaptic neuron to ACh is provided by the experiments, which examined the effects of the amines on ACh responses recorded from BA1 (6.3.3-4); bath application of 5HT ( $10^{-4}$  M) and dopamine ( $10^{-4}$  M) caused the amplitude of the ACh responses recorded from BA1 to decrease by 57 % and 14 %, respectively. This does not however rule out the possibility that the biogenic amines are also modulating cholinergic synaptic transmission by regulating ACh release from the presynaptic terminals of the fSR. Another study (Ramirez and Orchard, 1990) has reported that 5HT is capable of modulating the activity in the fSR, albeit through octopamine receptors.

#### **6.4.2. How do the amines mediate their modulatory effect?**

The amines may mediate their modulatory effects on BA1 via a number of different mechanisms. They may alter the membrane conductance of BA1 or they may act at receptors on BA1. Alternatively they may interfere with the second messenger systems in BA1, alter the ionic gradients across the membrane of BA1 or affect the number of cholinergic receptors available to bind ACh.

The input resistance and membrane potential of BA1 were monitored throughout the experiments presented in this study to determine if the amines alter the membrane conductance of BA1. The input resistance did not change significantly in the presence of the amines although the membrane potential did. Following the application of 5HT the membrane potential of BA1 depolarised in four preparations, while in 6 other preparations the membrane potential of BA1 hyperpolarised. The observation that 5HT

generally caused the membrane potential of BA1 to shift to approximately -65 mV, indicates that 5HT modulates an ionic conductance with a reversal potential of -65 mV in BA1. Following the application of dopamine the membrane potential of BA1 depolarised (1 - 14 mV) in this study, which is fairly consistent with the results from a study which examined the effects of dopamine on BA1 in the locust, *Schistocerca gregaria* (Prothero et al., 1995). Although 5HT and dopamine did cause changes in the membrane potential of BA1, it does not appear to be responsible for the decrease in the amplitude of the cholinergic responses recorded from BA1. The amplitude of the cholinergic responses recorded from BA1 decreased and remained suppressed following the application of 5HT and dopamine, regardless of whether the membrane potential of BA1 was depolarised or hyperpolarised, which suggests that the decrease in the cholinergic response of BA1 is due to a mechanism other than a change in the membrane conductance of BA1.

5HT and dopamine may mediate their modulatory effects on the cholinergic responses of BA1 by activating receptors which are coupled to second messenger pathways. These second messenger systems may modulate the cholinergic receptors responsible for the cholinergic response of BA1. A recent study (Reale et al., 1997) has reported that dopamine receptors cloned from *Drosophila* are coupled to two second messenger systems which are agonist specific; activation of the receptors stimulates an adenylate cyclase pathway and causes an increase in intracellular  $\text{Ca}^{2+}$  concentration. Evidence of a single class of dopamine receptor coupled to both an adenylate cyclase pathway and a pathway involving calcium has also been reported in a study on cockroach salivary gland acinar cells (Evans and Green, 1990). The majority of insect 5HT receptors and dopamine receptors, however, are reported to be coupled to the adenylate cyclase

pathway only (Nathanson and Greengard, 1973; Bodnaryk, 1979; Drummond et al., 1980; Berridge and Heslop, 1981; Uzzan and Dudai, 1982; Orchard et al., 1983; Cattell et al., 1985; Downer et al., 1985; Pratt and Pryor, 1986; Orr et al., 1987; Witz et al., 1990; Saudou et al., 1992). In the adenylate cyclase pathway, adenylate cyclase converts ATP to cAMP which in turn activates protein kinases which phosphorylate certain proteins to alter their shape and functions. The adenylate cyclase pathway is thought to be responsible for the 5HT-induced facilitation of ACh release from *Aplysia* siphon sensory neurons. Activation of 5HT receptors on the sensory neurons, which are positively coupled to adenylate cyclase, causes an increase in the cAMP levels, which activates protein kinases (Brunelli et al., 1976; Castellucci et al., 1980). The cAMP-dependent protein kinases phosphorylate and close specific S-type  $K^+$  channels (Klein et al., 1982; Siegelbaum et al., 1982; Abrams et al., 1984; Shuster et al., 1985), which increases the duration of the action potentials in the sensory neurons (Goldsmith and Abrams, 1992). This leads to an increase in  $Ca^{2+}$  influx, which ultimately enhances transmitter release from the sensory neurons (Klein and Kandel, 1980).

As the majority of invertebrate aminergic receptors mediate their effects via the adenylate cyclase pathway, it is possible that the activation of aminergic receptors on BA1 will stimulate cAMP-dependent protein kinases to phosphorylate the cholinergic receptors responsible for the cholinergic response of BA1 and, thereby, cause the response amplitude to decrease. Indeed, there is evidence in vertebrate preparations that neuronal cholinergic receptors can be phosphorylated and therefore modulated by protein kinases (Swope et al., 1995; Eilers et al., 1997; Hsu et al., 1997; Paradiso and Brehm, 1998; Fenster, 1999). Evidence that aminergic modulation of cholinergic responses is mediated via the adenylate cyclase pathway in invertebrate neurons is

provided in recent studies which examined the aminergic modulation of the cholinergic responses recorded from the cockroach  $D_f$  motoneuron (Butt and Pitman, 1998, 1999).

The membrane-soluble analogues of cAMP and cGMP mimic the effects of 5HT on nicotinic cholinergic responses recorded from  $D_f$  (Butt and Pitman, 1998).

Additionally, a protein kinase inhibitor reversibly suppresses the effects of 5HT on the nicotinic cholinergic responses, and a phosphatase inhibitor renders the effects of 5HT irreversible (Butt and Pitman, 1999). These results indicate that protein phosphorylation is required for the amine, 5HT, to modulate nicotinic cholinergic responses recorded from the  $D_f$  motoneuron in the cockroach. As there are similarities between the aminergic modulation of the cholinergic responses recorded from the cockroach  $D_f$  motoneuron and locust BA1 motoneuron (both are suppressed by 5HT and dopamine by a cellular mechanism other than a change in membrane conductance), it is possible that the nicotinic receptors on BA1 are phosphorylated by cAMP-dependent protein kinases stimulated by the activation of 5HT aminergic receptors on BA1.

#### **6.4.3. Source of the amines**

The results from the electrophysiological experiments presented in this chapter, clearly demonstrate that both 5HT and dopamine are potentially capable of modulating the fSR/BA1 synapse and may be candidates for the neurotransmitters released from the neurons (revealed in the EM ICC studies, Chapter 4) in synaptic or close contact with the fSR and cells to which it is presynaptic. Amines released from these neurons could potentially act on the fSR and/or BA1, inhibiting cholinergic synaptic transmission.

The presence of the amines in the dorso-lateral neuropil region, which contains the fSR/BA1 synapses, would indicate that the amines could at least potentially modulate

cholinergic synaptic transmission between the fSR and BA1. A previous study (Watson, 1992b) has shown dopamine-like IR in the dorso-lateral neuropil regions of the locust mesothoracic ganglion. A pair of large dopamine-IR axons, that run in the median dorsal tract (MDT), give rise to prominent branches that run along the dorsal edge of the mesothoracic ganglion both laterally and medially, and also to branches that extend ventrally that repeatedly subdivide. Large dopamine-IR axons located in the dorsal intermediate tract (DIT) give rise to long branches that extend dorsally and laterally. There are also fine dopamine-IR neurites that are evenly distributed throughout the entire mesothoracic ganglion. The presence of dopamine-IR in the dorso-lateral neuropil regions of the mesothoracic ganglion, indicates that dopamine is present in the region where it could potentially modulate the fSR/BA1 synapse. The dopamine-IR neurons revealed in other studies are mostly intersegmental interneurons that are present in all neuropil regions (Elekes et al., 1987; Watson, 1992b). It is possible therefore, that some of the neurons that make synaptic contact with the fSR, and cells to which it is presynaptic, are intersegmental interneurons that release dopamine. Additionally, dopamine-IR intersegmental interneurons have been reported to make dyadic outputs (Elekes et al., 1987). The majority of the neurons revealed in the EM ICC studies that are not immunoreactive for GABA or glutamate (see Chapter 4) make dyadic synapses onto the fSR and cells to which it is presynaptic.

As no previous study has examined the distribution of 5HT-IR in the locust mesothoracic ganglion, confocal microscopical ICC was used to determine if 5HT is present in aLAC, the dorso-lateral neuropil region into which the dendrites of the fSR and the flight motoneurons project. The distribution of 5HT-IR in the transverse sections taken through the mesothoracic ganglion revealed in this study correlates well

with the distribution of 5HT-IR in transverse sections through the locust metathoracic ganglion anterior to nerve 3 as identified in a study by Tyrer et al. (1984). This indicates the 5HT-IR revealed in this study is specific. Very intense Rhodamine red fluorescence was found in the ventral association centres (vVAC). The most intense staining was in the ventralmost VAC (vVAC), along the dorsal edge of the anterior VAC (aVAC) (Figure 6.8A) and in the lateral VAC (lVAC) (Figure 6.8B). Tyrer et al (1984) suggested that the 5HT-IR branches revealed elsewhere in the ganglion belonged to interneurons. In the present study 5HT-IR axons were also clearly seen running within the dorsal median tract (DMT) (Figure 6.8A) and in the ventral intermediate tract (VIT) (Figure 4.5A), an observation that was also made in the metathoracic ganglion by Tyrer et al., (1984). Interestingly, there was very little staining observed in the dorsal neuropil regions. This indicates that 5HT is either not present or present in low concentrations in the neuropil region where it could potentially modulate the fSR/BA1 synapse.

#### **6.4.4. Aminergic receptors**

The results presented in this chapter reveal that 5HT can modulate cholinergic synaptic transmission between the fSR and BA1 under experimental conditions but it does not appear to be present in high concentrations in the neuropil region where it could potentially modulate the fSR/BA1 synapse *in vivo*. This may indicate that 5HT is capable of modulating the cholinergic responses of BA1 via a receptor for another amine. The results from the experiments in this study indicate that 5HT shares a receptor or second messenger pathway with the biogenic amine, octopamine; pre-incubating with octopamine occludes the action of 5HT. A number of other studies on



the effects of amines on invertebrate neurons also suggest that 5HT and octopamine act at a common receptor (Evans, 1981; Whim and Evans, 1988; Ramirez and Orchard, 1990; Butt and Pitman, personal communication). Both 5HT and octopamine increase the relaxation rate of twitch tension in the locust extensor-tibiae muscle (Evans, 1981) and locust dorsal longitudinal flight muscles (Whim and Evans, 1988). They also increase the number and frequency of spikes in the locust fSR in response to wing movements (Ramirez and Orchard, 1990) and modulate the nicotinic cholinergic responses of the cockroach  $D_f$  motoneuron (Butt and Pitman, 1998). Co-application of 5HT and octopamine did not have a significant additive effect on the nicotinic cholinergic responses of the cockroach  $D_f$  motoneuron, indicating both amines act upon a common receptor in the cockroach, although the identity of the common receptor in  $D_f$  is unclear (Butt and Pitman, personal communication). The results from the other invertebrate studies, however, provide evidence for the identity of a common receptor. The effects of 5HT and octopamine on the locust extensor-tibiae muscle and locust dorsal longitudinal flight muscles (i.e. the increase in the relaxation rate of twitch tension) and also their effects on the locust fSR in response to wing movements (i.e. the increase in the number and frequency of spikes) are antagonised by the  $\alpha$ -adrenoreceptor antagonist, phentolamine (which is reported to block insect octopamine receptors [Evans, 1981; Roeder, 1992] ) but not by 5HT receptor antagonists (Evans, 1981; Whim and Evans, 1988; Ramirez and Orchard, 1990). These results indicate that 5HT may be acting as an agonist at an octopamine receptor. It is, therefore, also possible that 5HT is modulating cholinergic synaptic transmission between the fSR and BA1 via an octopamine receptor.

## Chapter 7. DISCUSSION

### 7.1. Modulation of the fSR/BA1 synapse in *Locusta migratoria*.

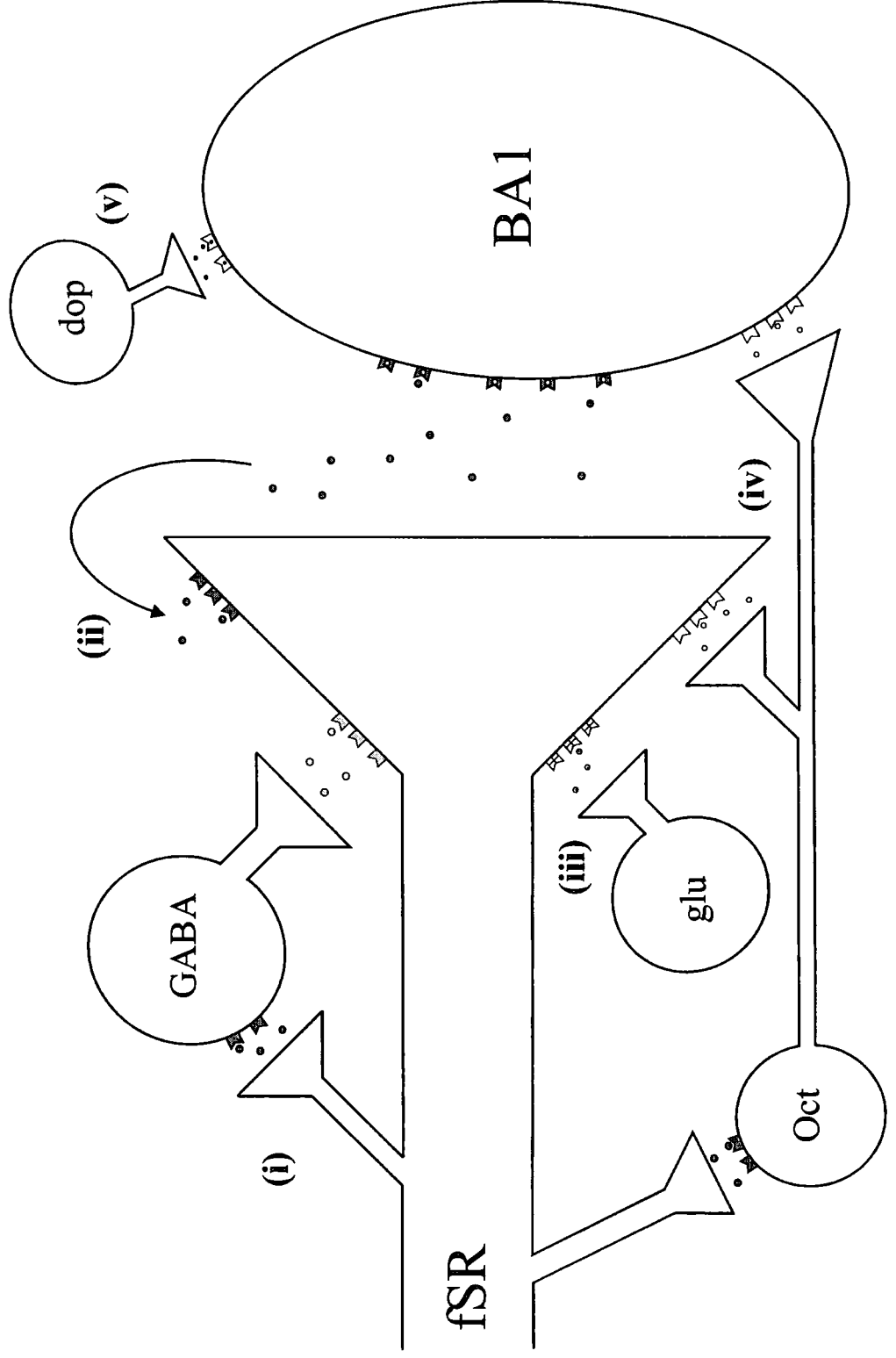
The results from the studies presented in this thesis indicate that a variety of neurotransmitters and neuromodulators are potentially capable of modulating cholinergic synaptic transmission between the fSR and BA1. ACh released from the fSR itself (Chapter 3), GABA and glutamate (Chapter 3,4) all appear to be involved in the presynaptic modulation of ACh release from the sensory neuron and there is evidence that octopamine (Chapter 5) and dopamine (Chapter 6) may be involved in the modulation of the responses of the postsynaptic neuron, BA1, to ACh (Figure 7.1).

The neuropharmacological experiments presented in Chapter 3 confirm that presynaptic muscarinic receptors in *Locusta migratoria* are involved in the modulation of ACh release from the presynaptic terminals of the fSR, which has been reported previously for *Schistocerca gregaria* (Leitch and Pitman, 1995). The results from further electrophysiological experiments indicate that at least some of these muscarinic receptors are located on feed-forward GABAergic interneurons that synaptically inhibit the fSR terminals (Figure 7.1i). These results are supported by a complementary EM ICC study, which reveals that the fSR does receive synaptic inputs from GABA-IR neurons (Chapter 4). It is also possible that some of the presynaptic muscarinic receptors are located on the fSR terminals themselves and act as autoreceptors (Figure 7.1ii). Evidence that the fSR terminals may also be presynaptically modulated by glutamate (Figure 7.1iii) was revealed in an additional EM ICC study, which showed that the glutamate-IR neurons are presynaptic to the fSR (Chapter 4). The results from

**Figure 7.1. Modulation of the fSR/BA1 synapse.** A diagram showing the neurotransmitters involved in modulating cholinergic synaptic transmission at the fSR/BA1 synapse based on the results in this study. Stimulation of the fSR axon, results in the release of acetylcholine (ACh) from the fSR terminals, which binds to nicotinic cholinergic receptors on BA1, evoking EPSPs (i) ACh released from a collateral of the fSR binds to muscarinic receptors on a GABAergic interneuron (GABA), which releases GABA to bind with the GABA receptors on the fSR terminal and inhibit ACh release. (ii) ACh released from the fSR terminal activates muscarinic autoreceptors on the fSR to inhibit ACh release (iii) Glutamate (glu) released onto the fSR terminals activates either excitatory or inhibitory glutamate receptors to modulate ACh release. (iv) ACh released from a collateral of the fSR binds to cholinergic receptors on an octopamine neuron (oct), which releases octopamine. The octopamine binds to inhibitory octopamine receptors on the fSR and BA1, causing a decrease in ACh release from the fSR and a decrease in the cholinergic response of BA1. (v) Dopamine (dop) released onto BA1 activates dopamine receptors, which suppress the cholinergic response of BA1.

Figure 7.1. Modulation of the fSR/BA1 synapse.

- Neurotransmitters**
- ACh
  - GABA
  - glutamate
  - octopamine
  - dopamine
- Receptors**
- muscarinic
  - nicotinic
  - GABA
  - glutamate
  - octopamine
  - dopamine



electrophysiological experiments presented in Chapter 5, indicate that octopamine is potentially capable of modulating the fSR/BA1 synapse. The results show that octopamine modulates synaptic transmission between the fSR and BA1 by modifying the cholinergic response of the postsynaptic neuron, BA1 (Figure 7.1iv). However, there is also evidence that octopamine may act on a different octopamine receptor subtype to presynaptically modulate the fSR. The biogenic amine, dopamine is also potentially capable of modulating the fSR/BA1 synapse by modifying the response of BA1 to ACh (Figure 7.1v) but the results presented in Chapter 6 indicate that the effects of 5HT may be mediated via octopamine receptors.

Many of the results presented in this thesis are from neuropharmacological electrophysiological studies, in which the effects of various agonists and antagonists on cholinergic synaptic transmission at the fSR/BA1 synapse were examined. One of the problems with this type of study is that many of the test compounds used may not be specific for a particular receptor type. For example, high concentrations of muscarinic receptor antagonists are reported to block insect nicotinic cholinergic receptors (Shankland et al., 1971; Schmidt-Nielsen et al., 1977; Hue et al., 1989; David and Sattelle, 1984; Trimmer and Weeks, 1989) and the GABA antagonist picrotoxin is also reported to block nicotinic cholinergic (Benson, 1992, 1993) and glutamate receptors (Cull-Candy, 1976; Wafford and Sattelle, 1989; Washio, 1994; Etter et al., 1999; Duan and Cooke, 2000). The reason that many of the compounds act at different receptor types is due to structural and phylogenetic similarities between the receptors. GABA, glutamate and nicotinic receptors, for example, are all ligand-gated ion channels that belong to a gene superfamily (Betz, 1990) that share a common three-dimensional structure (Olsen and Tobin, 1990; Karlin, 1993; Cully et al., 1996). Discrimination of

partial selectivity can be achieved to some extent by using lower concentrations of the compounds, as some receptors are less sensitive than others to certain compounds. For example, picrotoxin ( $10^{-6}$  M) completely blocks GABA responses on the cockroach  $D_f$  motoneuron but does not affect glutamate responses (Wafford and Sattelle, 1989). Another way is to use a range of compounds reported to act at a particular receptor type (e.g. octopamine) and assess all their affects.

Some of the conclusions drawn from the neuropharmacological electrophysiological studies presented in this thesis are also based on the assumption that the extrasynaptic receptors on the cell body of BA1 are the same as the synaptic receptors on the postsynaptic membrane of BA1 within the neuropil. There is some evidence to suggest, however, that receptors on the cell body of insect neurons may be different from insect synaptic receptors (see Benson, 1993; Dubreil et al., 1994). Therefore the effects picrotoxin has on fSR-evoked EPSPs recorded from BA1 could be due to picrotoxin increasing the sensitivity of BA1 to ACh at the synapse, even though picrotoxin does not cause an increase the sensitivity of the cell body of BA1 to locally-applied ACh. To establish if the test compounds do modulate BA1 at the fSR/BA1 synapse, the test compounds would have to be injected directly into the neuropil, which is technically difficult. Another drawback to the bath application of the test compounds is the possibility that the receptors will become desensitised. However, there is no evidence that the effects of the test compounds on synaptic transmission at the fSR/BA1 synapse are due to the desensitisation of the receptors involved in evoking and modulating transmission.

Evidence that the test compounds modulate the presynaptic afferent fSR can be provided more easily by double-labelling EM studies. For example, the suggestion that picrotoxin increases synaptic transmission between the fSR and BA1, by blocking presynaptic GABA receptors on the fSR that normally inhibit ACh release is supported by the results from the complementary GABA-ICC study (Chapter 4). However, caution must also be exercised in interpreting the results from ICC studies. Immunolabelling with an antibody will demonstrate that a neuron presynaptic to the fSR contains a particular neurotransmitter but it does not necessarily mean that that neurotransmitter is released onto the fSR. There is evidence that a number of neurotransmitters are co-localised in the same neurons (Keshishian and O'Shea, 1985; Distler, 1990; Stevenson, 1998), so it is possible that the neurotransmitter released from a neuron is different to the neurotransmitter shown to be present by ICC. However, if complementary electrophysiological studies support the ICC findings, as is in the case of the GABAergic modulation of the fSR, it will strongly indicate that the neurotransmitter shown to be present in a neuron by immunolabelling, is the same one released from the neuron.

Therefore, after considering the results presented in this thesis in light of the drawbacks with the methods used, the conclusions are the same. ACh, GABA, glutamate, octopamine, 5HT and dopamine all appear to be potentially capable of modulating cholinergic synaptic transmission at the fSR/BA1 synapse.

## 7.2. Interactions between neurotransmitters

Transmission at other synapses has also been shown to be modulated by a variety of neurotransmitters and neuromodulators. Glutamatergic transmission at the central synapses between the locust FETi and flexor tibiae motoneurons (Parker, 1994, 1995, 1996) and between the neurons in the lobster pyloric system (Johnson and Harris-Warrick, 1997) is modulated by the biogenic amines octopamine, 5HT and dopamine. Likewise, the biogenic amines octopamine, 5HT and dopamine modulate cholinergic synaptic transmission at the synapses between the neurons in the cockroach escape response pathway (Casagrand and Ritzmann, 1992a; Butt and Pitman, 1998) and the neurons in the lobster pyloric system (Johnson and Harris-Warrick, 1997). Different amines have opposite effects on synaptic transmission at these synapses and the effects the amines have, varies depending on which synapse they are modulating. At the cockroach vGI/thoracic interneuron synapses, for example, octopamine and dopamine enhance cholinergic synaptic transmission, whereas 5HT reduces it (Casagrand and Ritzmann, 1992a). However, the cholinergic responses of the cockroach D<sub>r</sub> motoneuron, which is part of the same neuronal pathway as the vGI/thoracic interneuron synapses, are reduced by octopamine, dopamine and 5HT (Butt and Pitman, 1998).

The variation in the modulation of synaptic transmission is not only dependent on the types of neuromodulators which are involved, but on the receptors at which the neuromodulators act. It has been reported in both invertebrate and vertebrate preparations that different classes of receptors activated by the same neurotransmitter/modulator have excitatory and inhibitory effects (Howells and Evans, 1998; Tellez et al., 1999). At cholinergic synapses, for example, postsynaptic nicotinic



cholinergic receptors may be responsible for the excitatory responses evoked by ACh in the postsynaptic neuron (Sattelle et al., 1983; Parker and Newland, 1995; Leitch and Pitman, 1995), whereas presynaptic muscarinic cholinergic receptors may be responsible for down-regulating ACh release (Hue et al., 1989; Knipper and Breer, 1989; Trimmer and Weeks, 1989; Leitch and Pitman, 1995; Parker and Newland, 1995). It has been suggested that fast postsynaptic nicotinic cholinergic receptors and slow presynaptic muscarinic cholinergic receptors at the synapses between the locust retinotopic unit afferents and lobula giant movement detector (LGMD) neuron may allow ACh to both excite the LGMD and inhibit neighbouring afferents (Rind and Simmons, 1998).

The cellular mechanisms that are evoked by the various neurotransmitters/modulators to modulate cholinergic synaptic transmission at the fSR/BA1 synapse are unclear at this present time although there are a number of possibilities. The results indicate that the biogenic amines, octopamine, 5HT and dopamine modulate the fSR/BA1 synapse (at least in part) by reducing the responses of the postsynaptic neuron, BA1 to ACh. As the majority of invertebrate receptors for the biogenic amines are coupled to adenylate cyclase (Nathanson and Greengard, 1973; Bodnaryk, 1979; Nathanson, 1979; Drummond et al., 1980; Berridge and Heslop, 1981; Deterre et al., 1982; Uzzan and Dudai, 1982; Orchard et al., 1983; Evans 1984 a,b; Stoof et al., 1984; Cattell et al., 1985; Downer et al., 1985; ; de Vlieger et al., 1986; Pratt and Pryor, 1986; Orr et al., 1987; Orr and Hollingworth, 1990; Witz et al., 1990; Orr et al., 1991; Kaupp et al., 1992; Saudou et al., 1992; Lange and Tsang, 1993; Roeder and Nathanson, 1993; Baines and Downer, 1994; Li et al., 1994; Parker, 1995; Han et al., 1998; Butt and Pitman, 1999) it is likely that the aminergic receptors on BA1 are also coupled to

adenylate cyclase. Recent studies (Butt and Pitman, 1998, 1999) have provided evidence that aminergic receptors on the  $D_f$  motoneuron in the cockroach modulate the nicotinic cholinergic responses of  $D_f$  via an adenylate cyclase pathway; the membrane-soluble analogues of cAMP and cGMP mimic the effects of 5HT, a protein kinase inhibitor reversibly suppresses the effects of 5HT and a phosphatase inhibitor renders the effects of 5HT irreversible (Butt and Pitman, 1998, 1999). As there are similarities between the aminergic modulation of the cholinergic responses recorded from the cockroach  $D_f$  motoneuron and locust BA1 motoneuron (both are suppressed by octopamine, 5HT and dopamine by a cellular mechanism other than a change in membrane conductance), it is possible that the nicotinic receptors on BA1 are modulated via an adenylate cyclase stimulated by the activation of aminergic receptors on BA1. However, further work needs to be completed using membrane-soluble analogues of cAMP and cGMP and protein kinase and phosphatase inhibitors, to establish if the aminergic modulation of the nicotinic cholinergic responses of BA1 is mediated via an adenylate cyclase pathway.

GABA and glutamate on the other hand, appear to modulate the fSR/BA1 synapse by modulating ACh release from the fSR. It is probable that activation of the GABA receptors on the fSR, evokes a hyperpolarising  $Cl^-$  conductance. If ACh is released from the fSR according to the  $Ca^{2+}$  hypothesis (i.e. a depolarisation opens  $Ca^{2+}$  channels, causing a  $Ca^{2+}$  influx which triggers transmitter release), hyperpolarising the presynaptic terminals of the fSR would shorten the depolarising action potentials, leading to a decrease in  $Ca^{2+}$  influx and a decrease in transmitter release. Similar intracellular events may also be evoked if the glutamate receptors on the fSR are

ligand-gated chloride channels (Wafford and Sattelle, 1989; Dubas, 1990; Pearlstein et al., 1994; Washio, 1994).

On the other hand, the glutamate receptors on the fSR may be excitatory G-protein-coupled receptors positively coupled to adenylate cyclase (Levi et al., 1999).

Stimulation of this type of receptor may activate a chain of intracellular events similar to those evoked by adenylate cyclase-coupled 5HT receptors on the *Aplysia* siphon sensory neurons (Klein and Kandel, 1980) and FLRFamide receptors on *Aplysia* buccal ganglion neurons (Baux et al., 1993), which ultimately leads to an increase in  $\text{Ca}^{2+}$  influx and an increase in transmitter release.

Presynaptic muscarinic cholinergic receptors are also involved in modulating ACh release from the fSR, and the results from Chapter 3 indicate that at least some of the presynaptic muscarinic are located on GABAergic interneurons, which inhibit the fSR. However, this does not rule out the possibility that muscarinic autoreceptors are also located on the fSR. The cellular mechanisms evoked when insect presynaptic muscarinic autoreceptors are stimulated are unknown, although it is possible the receptors are negatively coupled to adenylate cyclase as the activation of muscarinic autoreceptors on locust synaptosomes significantly reduces the accumulation of cAMP (Knipper and Breer, 1988). It had previously been thought that neuromodulators inhibited neurotransmitter release by inhibiting  $\text{Ca}^{2+}$  channels and activating  $\text{K}^{+}$  channels, which shorten action potentials. Recent studies, indicate that the intracellular mechanisms evoked by presynaptic receptors are more complex (Baux et al., 1993; Wu and Saggau, 1997; Slutsky et al., 1999; Parnas et al., 2000). Inhibition of  $\text{Ca}^{2+}$  influx (e.g. by second messengers phosphorylating the calcium channels) does

contribute to the presynaptic inhibition of ACh release (see Wu and Saggau, 1997) but it does not entirely account for presynaptic inhibition. For example, activation of M<sub>2</sub> muscarinic receptors at the frog neuromuscular junction inhibits ACh release but it does not affect the presynaptic Ca<sup>2+</sup> currents (Slutsky, et al., 1999). It has been suggested that other mechanisms such as the direct inhibition of the release machinery by the second messengers of the presynaptic receptors (see Wu and Saggau, 1997, Parnas, 2000), are also involved.

### **7.3. Behavioural significance**

The locust fSR/BA1 synapse was used as a model in this thesis to examine the modulation of cholinergic synaptic transmission between a single identified sensory afferent and an identified motoneuron. The results indicate that ACh, GABA, glutamate, octopamine and dopamine are all potentially capable of modulating the synapse (see Figure 7.1). This does not necessarily mean however, that the synapse is modulated by this variety of modulators *in vivo*. Under the experimental conditions in this study, the fSR was stimulated electrically at a rate of 0.1 Hz whereas, the fSR in an adult locust would be stimulated a rate of 23 Hz by wing movement in normal flight (Gray and Robertson, 1994), with each wing beat producing up to 15-20 spikes (Möhl, 1985). Therefore the effects the neuromodulators have on the synapse under experimental conditions may be different from the effects they have *in vivo*. In addition, the experiments were performed on an isolated ganglia preparation rather than an intact animal, which could potentially alter the effects the neuromodulators have on the fSR/BA1 synapse. This may not be a problem, however, as it was reported in another study (Ramirez and Orchard, 1990) that there was no difference in the effects of

octopamine on the fSR recorded from an isolated ganglia preparation or an intact animal.

Another problem to be aware of is the possibility that the biogenic amines, which modulate the synapse when bath applied under experimental conditions (Chapter 5,6), may not be present in dorso-lateral neuropil region containing the synapses between the fSR and BA1. The confocal microscopical ICC study using an anti-5HT antibody, revealed the dorso-lateral neuropil region containing the synapses between the fSR and BA1, showed a very low level of 5HT-immunoreactivity. This suggests that 5HT may not modulate the fSR/BA1 synapse under normal conditions and is consistent with the results from further electrophysiological experiments, which indicate that 5HT modulation of the synapse under experimental conditions, may be through octopamine receptors.

It is possible therefore, that under normal physiological conditions the fSR/BA1 synapse is not modulated by the variety of neuromodulators demonstrated in this thesis. However, there is strong evidence to suggest that all these neuromodulators do play an important role in insect flight (Robertson and Pearson, 1983; Claassen and Kammer, 1986; Pearson and Robertson, 1987; Reye and Pearson, 1987; Bicker et al., 1988; Robertson and Wisniowski, 1988; Dubas, 1990, 1991; Orchard et al., 1993; Witten and Truman, 1998) and as the fSR co-ordinates and regulates the flight motor pattern (Pearson et al., 1983; Reye and Pearson, 1988; Gray and Robertson, 1997) it is possible the neuromodulators act at the fSR/BA1 synapse to allow fine adjustments to made to the flight motor pattern, which is essential to control and maintain flight.

To conclude, the results from this study indicate that cholinergic synaptic transmission between the locust fSR and BA1 is inhibited (at least in part) by a GABAergic feed-forward loop activated via presynaptic muscarinic receptors, which inhibits ACh release from the fSR. ACh release may also be modulated by glutamate-IR neurons shown in the ICC study. The biogenic amines, octopamine and dopamine are also involved in modulating cholinergic synaptic transmission at the fSR/BA1 synapse and the results indicate that they do this (at least in part) by suppressing the responses of BA1 to ACh. Therefore, this study provides conclusive evidence that a range of neuromodulators and neurotransmitters interact to modulate synaptic transmission.

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## **Appendices**

### **Appendix 1. Timms staining solution.**

1. Heat 100 ml deionised distilled water (DDW) to 50 °C and add 3g gum acacia and quickly stir.
2. After it has dissolved add 10g sucrose, 0.43g citric acid and 0.17g hydroquinone. Continue to warm and stir until everything has dissolved.
3. Filter the solution warm and change the filter paper often. Store in fridge.

Bacon and Altman (1977).

### **Appendix 2. Soft araldite resin.**

1. Warm 13 ml Araldite CY 212 (Agar Aids, Stansted, U.K.) and 7 ml D.D.S.A. (Agar Aids, Stansted, U.K.) to 45 °C.
2. Stir and add 0.4 ml B.D.M.A. (Agar Aids, Stansted, U.K.) and 1 ml of dibutyl phthalate (Agar Aids, Stansted, U.K.).

### **Appendix 3. Tris Buffer.**

1. 0.2M Tris Buffer was made by dissolving 2.42g Tris (mwt 121.g) in 100 ml DDW and adjusting the pH to 7.4.

### **Appendix 4. Sorensons phosphate buffer.**

1. A stock solution of 0.2 M solution of dibasic sodium phosphate was prepared by adding 35.61g of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  to 1l of DDW.
2. A stock solution of 0.2 M solution of monobasic sodium phosphate was prepared by adding 27.6g of  $\text{Na.H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  to 1l of DDW.
3. A 0.2M solution of Sorenson phosphate buffer pH 7.2. was made by mixing 36 ml of the dibasic solution with 14 ml of the monobasic solution. The pH of the buffer was measured to check it was correct but if it was not the appropriate acid or alkaline was added to adjust the pH.
4. To make a 0.1M solution of Sorensons phosphate buffer with 0.2M sucrose, 3.4g of sucrose was dissolved in 50 ml of 0.2M Sorensons phosphate buffer and 50 ml DDW.

### **Appendix 5. Incubation medium containing diaminobenzidine.**

1. Dissolve 20 mg ammonium chloride and 100 mg  $\beta$ -D-glucose in 50 ml 0.1M Sorensons phosphate buffer without sucrose.
2. Add 5 mg diaminobenzidine (1 ml 0.5% solution) to 9 ml incubation medium.

#### **Appendix 6. Bovine serum albumin/Tris Buffer.**

1. Dissolve 0.242g Tris (mwt 121g), 0.9g NaCl and 0.1g bovine serum (room temperature) in 100 ml DDW.
2. Adjust pH to 8.2 and bottle 50 ml before adjusting pH to 7.4.
3. Add 0.5 ml Tween 20 to each 50 ml bottle and mix.

#### **Appendix 7. Reynolds lead citrate.**

1. Add 1.33g lead nitrate, 1.76g sodium citrate, 30 ml DDW to a 50 ml flask.
2. Shake vigorously for 1 minute and intermittently for a further 30 minutes.
3. Add 8 ml 0.1 M NaOH and invert the flask.
4. Make up to 50 ml with DDW.

